The gut microbiota in cardiovascular disease

Interactions between the diet, microbiota and the gut immune barrier

Eelke Brandsma

Research
The research described in this thesis was performed in the department of Pediatrics, section Molecular Genetics at the University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands.

Funding
The research described in this thesis was funded by a grant from Cardio-Vasculair Onderzoek Nederland (CVON2012-03) and by the Jan Kornelis de Cock Foundation.

rijksuniversiteit groningen

The gut microbiota in cardiovascular disease

Interactions between the diet, microbiota and the gut immune barrier

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 27 februari 2019 om 12.45 uur

door

Eelke Thijs Brandsma

geboren op 16 juli 1987
te Heerenveen

Promotor

Dr. ing. A.J.A. van de Sluis

Copromotor

Dr. D.P.Y. Koonen

Beoordelingscommissie

Prof. dr. K.N. Faber
Prof. dr. A.K. Groen
Prof. dr. L. Joosten

Table of contents

CHAPTER 1

Introduction

Introduction

Cardiovascular disease (CVD) is a major burden for the society worldwide leading to 17.7 million deaths per year and causing 31% of all global deaths. The majority of cardiovascular deaths is caused by heart attacks and strokes with atherosclerosis as the main underlying cause (World Health Organization). Atherosclerosis starts by the formation of fatty streaks, which are formed by the deposition of lipids and cholesterol and the infiltration of blood leukocytes into the intima of the vessel wall. Consequently, the infiltrated monocytes differentiate into macrophages, which take up lipids via scavenger receptors to form foam cells. Smooth muscle cells (SMC) infiltrate from the media into the intima to form the fibrous cap. Cell death of SMC and foam cells leads to extracellular lipid accumulation and formation of the necrotic core (Libby et al., 2011). Rupture of the fibrous cap can cause thrombus formation, which can block the blood flow of coronary or carotid arteries leading to myocardial infarction or a stroke respectively. Therefore, prevention and adequate treatment of atherosclerosis is essential to reduce the number of cardiovascular deaths worldwide.

Treatment of cardiovascular disease

In 1913, Anitschkov discovered the importance of dietary cholesterol in the atherogenic process (Anitschkov et al., 2011) and over the last century the importance of hyperlipidemia as a driving force for atherosclerosis has become well-established (Dawber et al., 1951; Ference et al., 2017; Gofman et al., 2007). The discovery of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, better known as statins, by Akiro Endo in 1976 was a major step in cardiovascular disease management (Endo et al., 1976a; Endo et al., 1976b). Inhibition of HMG-CoA reductase by statins affects cholesterol metabolism by preventing endogenous cholesterol synthesis, promoting uptake and degradation of plasma LDL-cholesterol and inhibiting scavenger receptors (Stancu and Sima, 2001) (Figure 1). This leads to a successful reduction of plasma LDL-cholesterol levels and a reduction in cardiovascular death (Group, 1994). The use of genetic linkage analysis has led to the discovery of new target genes for treatment of cardiovascular disease, including proprotein convertase subtilisin/kexin type 9 (PCSK9) (Abifadel et al., 2003). PCSK9 is an important modulator of the LDL receptor, increased

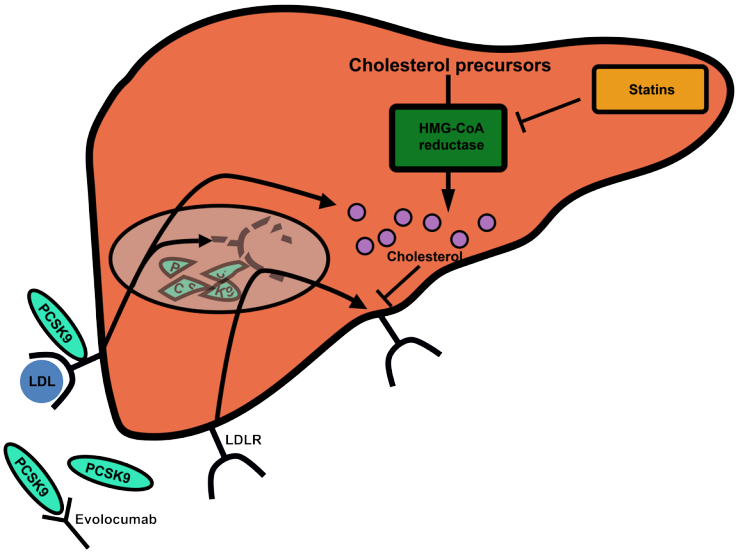


Figure 1. Mechanisms of cholesterol lowering medications
HMG-CoA reductase promotes endogenous cholesterol production by converting cholesterol precursors into cholesterol leading to increased intracellular cholesterol levels. An increase in intracellular cholesterol activates a negative feedback loop and decreases the expression of the LDL receptor, leading to decreased LDLR on the cell surface. Statins inhibit HMG-CoA reductase leading to decreased endogenous cholesterol production, increased LDLR expression on the cell surface and increased uptake and breakdown of LDL-cholesterol from the circulation, thereby reducing LDL-cholesterol levels in the circulation. PCSK9 tags the LDL receptor for degradation in the hepatocyte leading to decreased LDLR expression on the cell surface and decreased uptake of LDL-cholesterol from the circulation into the hepatocyte. The anti-PCSK9-antibody Evolocumab binds to PCSK9 in the circulation, thereby preventing the breakdown of LDL receptors on the surface of hepatocytes, leading to increased LDLR on the cell surface and increased uptake and breakdown of LDL-cholesterol from the circulation.

levels of PCSK9 in blood lead to downregulation of the LDL receptor in the liver, hence LDL-cholesterol levels rise in the circulation (Bjorklund et al., 2014). Recent studies have shown a 60% decrease in LDL-cholesterol on top of statin treatment following treatment with the PCSK9-inhibitor Evolocumab (Sabatine et al., 2017). Combined treatment by statins and PCSK9-inhibitors leads to a reduction of LDL-cholesterol levels to 0,78 mmol/L, which is well within the normolipidemic range (Sabatine et al.,

2017). Nevertheless, these strategies do not lead to complete protection against atherosclerosis. Combining statins and PCSK9-inhibitors, which successfully protect against hyperlipidemia, reduces the number of cardiovascular events by 50% (Sabatine et al., 2017), indicating that factors independent of hyperlipidemia contribute substantially to atherosclerosis. Next to hyperlipidemia, associative studies in human and mechanistic studies in mice support an important role for inflammation in atherosclerosis (Libby et al., 2011; Moss and Ramji, 2016; Ramji and Davies, 2015). This is supported by the CANTOS-trial, which investigated the efficacy of Canakinumab, an anti-inflammatory drug that targets the pro-inflammatory cytokine IL-1β. In the CANTOS-trial patients with previous myocardial infarction and increased systemic inflammation were treated with Canakinumab (Ridker et al., 2017). The study showed a significant reduction of 15% in the number of cardiovascular events following treatment with Canakinumab on top of statin treatment, thereby showing that targeting inflammation in atherosclerosis is therapeutically effective in the protection against cardiovascular disease.

Inflammation in atherosclerosis

Leukocytosis is associated with atherosclerosis in humans and specifically increased numbers of circulating monocytes and neutrophils are well-known to contribute to atherogenesis in *Ldlr-/-* and ApoE-/- mice (Swirski and Nahrendorf, 2013). Accumulation of cholesterol into hematopoietic and progenitor cells (HSPCs) leads to the production of GM-CSF and increased expression of the beta chain of the IL-3 receptor (IL-3Rβ) thereby promoting increased hematopoiesis leading to leukocytosis (Murphy et al., 2011; Yvan-Charvet et al., 2010). Circulating monocytes and neutrophils can consequently migrate into the intima of the vessel wall by binding to the adhesion molecules ICAM-1 and VCAM (Radi et al., 2001). In healthy conditions arterial epithelial cells resist binding of leukocytes, however dyslipidemia, hypertension or pro-inflammatory stimuli can upregulate the expression of ICAM-1 and VCAM thereby facilitating the uptake of leukocytes into the intima of the vessel wall (Libby et al., 2011). Following migration into atherosclerotic lesion areas neutrophils promote oxidative stress which leads to endothelial cell dysfunction, lesion growth and plaque instability (Swirski and Nahrendorf, 2013). In addition, neutrophils also stimulate further migration of monocytes into the lesion

area (Drechsler et al., 2010). Following migration of monocytes into the intima of the vessel wall monocytes can differentiate into macrophages (Libby et al., 2011). Intracellular lipid accumulation into macrophages via uptake of lipids transforms these macrophages into foam cells (Libby et al., 2011). Production of pro-inflammatory cytokines including IL-1β and TNF-α by macrophages progresses atherogenesis (Chi et al., 2004; Elhage et al., 1998; Kirii et al., 2003) and facilitates the infiltration of other leukocytes, including T-cells. Although the number of T-cells in atherosclerotic plaques is low, specific subsets do seem to be important in atherogenesis (Swirski and Nahrendorf, 2013). T-helper 1 and T-helper 17 cells are important producers of IFN-γ and IL-17 respectively and promote atherogenesis, whereas regulatory T-cells protect against atherosclerosis development by producing the anti-inflammatory cytokines IL-10 and TGF-β. Deficiency of the Th1 cytokine IFN-γ or the Th17 cytokine IL-17 reduces atherogenesis whereas administration of the anti-inflammatory cytokine IL-10 protects against atherosclerosis (Ramji and Davies, 2015). To summarize, atherosclerosis is associated with increased circulating numbers of leukocytes and specific leukocyte subsets, including monocytes, neutrophils and T-helper cells infiltrate into the atherosclerotic lesion area and produce pro-inflammatory cytokines and reactive oxygen species, altogether this further exacerbates atherogenesis.

Hyperlipidemia is one of the driving forces of the inflammatory process, nevertheless the anti-inflammatory drug Canakinumab effectively reduced the number of cardiovascular events by 15% during normolipidemic conditions (Ridker et al., 2017). This indicates that factors independent of hyperlipidemia contribute to the inflammatory process in the etiology of atherosclerosis. Although the CANTOS-trial effectively reduced cardiovascular events, an important side-effect was the increased incidence of fatal infections or sepsis. Increased susceptibility for infections is an intrinsic risk factor of drugs targeting inflammation, therefore it is important to understand which factors contribute to systemic inflammation in the atherogenic process. A better understanding of the mechanisms leading to systemic inflammation in the context of atherosclerosis development could lead to new therapeutic targets, where factors causing systemic inflammation could be directly targeted instead of the inflammatory process itself.

The microbiota in cardiovascular disease

Recently, the gut microbiota was identified as an additional player affecting atherosclerosis development (Koeth et al., 2013; Li et al., 2016; Tang and Hazen, 2014). Atherosclerosis is associated with changes in microbiota composition (Emoto et al., 2016; Jie et al., 2017; Karlsson et al., 2012). The presence of Collinsella in the gut microbiota of patients with symptomatic atherosclerosis is increased, whereas a decrease is observed for Eubacterium and Roseburia (Karlsson et al., 2012). In addition, a large case control study confirmed the reduction of Roseburia intestinalis and identified novel associations between gut microbiota composition and atherosclerosis, including a reduction in Faecalibacterium Prauznitzii and increased abundance of Ruminococcus Gnavus, Escherichia Coli, Klebsiella and Enterobacter aerogenes in CVD patients (Jie et al., 2017). Increased presence of Ruminococcus Gnavus was previously associated with inflammatory bowel disease and intestinal barrier function (Matsuoka and Kanai, 2015). A decrease in intestinal barrier function is associated with leakage of bacteria or endotoxins (e.g lipopolysaccharide, LPS) from the gut into the systemic circulation (Brandsma et al., 2015). In addition, bacteria or endotoxins can translocate from the gut into systemic circulation via leakage between intestinal epithelial cells or via active cellular transport, to be discussed in depth in **Chapter 2** and **Chapter 4**. Interestingly, taxonomies of the gut microbiota have been detected in atherosclerotic lesions (Koren et al., 2011) and systemic infection with Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis has been shown to promote systemic inflammation and atherogenesis (Hayashi et al., 2011; Zhang et al., 2010). Moreover, translocation of LPS from the gut lumen into the systemic circulation has been shown to promote systemic inflammation and atherogenesis (Li et al., 2016). Thus, translocation of bacteria or bacterial components (e.g LPS) from the gut into the systemic circulation may contribute to systemic inflammation and atherogenesis. In addition, the microbiota may also affect development of cardiovascular disease through systemic immune responses. Mono-colonizing germfree mice with 52 different bacteria increased or decreased the percentage of regulatory T-cells and macrophages in the colon as well as in systemic lymphoid organs (e.g spleen)(Geva-Zatorsky et al., 2017). A correlation of the frequencies of these immune cell subsets in the colon and systemic lymphoid organs suggests that gut bacteria may locally affect expansion

of immune cells in the colon, which consequently migrate to the systemic circulation (Geva-Zatorsky et al., 2017). Furthermore, the gut microbiome is associated with an altered ex-vivo cytokine production by peripheral blood mononuclear cells, indicating a relationship between the gut microbiome and systemic cytokine responses (Schirmer et al., 2016). A role for the gut microbiome in the regulation of the systemic immune system is further supported by the capacity of the gut microbiome to control hematopoiesis in primary immune sites, including the bone marrow (Khosravi et al., 2014). Altogether, the gut microbiome affects systemic immune cell populations, systemic cytokine responses and hematopoiesis, which have all been implicated in the development of cardiovascular disease.

Gut microbiota metabolites in inflammation and CVD

Next to translocation of bacteria or endotoxins, production of metabolites by the gut microbiome may also affect cardiovascular disease. The gut microbiota is important for the production of short-chain fatty acids (SCFA). SCFA are produced by the fermentation of dietary fibers by the gut microbiota, and acetate, butyrate and propionate are the most prominent SCFA (Besten et al., 2013). Interestingly, CVD mortality is negatively correlated with consumption of dietary fibers (Sahyoun et al., 2006) and a reduction in SCFA producing bacteria (e.g Roseburia intestinalis) has been observed in CVD patients (Karlsson et al., 2012). Furthermore, administration of butyrate reduces atherogenesis in ApoE-/- mice (Aguilar et al., 2014). Altogether this indicates a protective effect for the microbiota derived SCFA in atherogenesis. SCFA can be taken up from the intestinal lumen by enterocytes and can consequently be transported basolaterally to reach the systemic circulation where they can affect metabolic function (discussed in **Chapter 2**) and inflammation. The SCFA receptor GPR43 is highly expressed on leukocytes and acetate, butyrate and propionate all suppress NF-κB activity (Cox et al., 2009; Tedelind et al., 2007). Consequently this leads to a reduction in multiple pro-inflammatory cytokines, including several cytokines affecting atherogenesis, such as IFN-γ, IL-1β and IL-2 (Aoyama et al., 2010; Cavaglieri et al., 2003; Cox et al., 2009). Thus, changes in the capacity of the microbiota to produce the anti-inflammatory SCFA may influence systemic inflammation and atherogenesis.

Thesis aim and outline

Recent studies have linked microbiota composition to cardiovascular disease and inflammation. It is however unknown whether alterations in microbiota composition can contribute to cardiovascular disease development and if interactions between the gut immune barrier, diet and gut microbiota can affect systemic inflammation and atherogenesis. Therefore, we aimed to understand how the interaction between the diet, gut microbiota and intestinal immune barrier contributes to systemic inflammation and atherosclerosis.

Chapter 2 describes in depth how the gut microbiota is controlled by the mucosal immune system and how aberrancies in the control of the gut microbiota affects development of non-alcoholic fatty liver disease, type 2 diabetes and cardiovascular disease. In **Chapter 3** we investigated the effect of a pro-inflammatory gut microbiota on the development of systemic inflammation and atherosclerosis. Furthermore, we explored multiple mechanisms to understand how the pro-inflammatory microbiome of *Caspase1*$^{-/-}$ mice may affect atherosclerosis development. These questions were studied by transplanting the gut microbiota of *Caspase1*$^{-/-}$ mice into *Ldlr*$^{-/-}$ mice. **Chapter 4** explores the role of the antimicrobial peptide REG3y in the development of atherosclerosis. We investigated the development of atherosclerosis in Reg3y$^{-/-}$ mice to understand whether infiltration of the intestinal epithelial barrier by bacteria from the gut microbiota naturally residing in the lumen is involved in atherosclerosis development. **Chapter 5** discusses the role of western type diets in the development of type 2 diabetes. Here we aimed to provide better insight into the effect of high fat diet (HFD) feeding and dietary cholesterol on the intestinal epithelial barrier and how this relates to the development of type 2 diabetes. **In Chapter 6** we will discuss the major findings of this thesis and put them into the context of the field. Furthermore, we will discuss the current status and future perspectives for microbiota research in the field of cardiovascular diseases.

References

Anitschkow,N.N. and Chalatov,S. (1913). Ueber experimentelle Choleserinsteatose und ihre Bedeutung fur die Entstehung einiger pathologischer Prozesse. Zentralbl Allg Pathol 24:1-9

Abifadel, M., Varret, M., Rabès, J.-P., Allard, D., Ouguerram, K., Devillers, M., Cruaud, C., Benjannet, S., Wickham, L., Erlich, D., et al. (2003). Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat. Genet. 34, 154–156.

Aguilar, E.C., Leonel, A.J., Teixeira, L.G., Silva, A.R., Silva, J.F., Pelaez, J.M.N., Capettini, L.S.A., Lemos, V.S., Santos, R.A.S., and Alvarez-Leite, J.I. (2014). Butyrate impairs atherogenesis by reducing plaque inflammation and vulnerability and decreasing NFκB activation. Nutr. Metab. Cardiovasc. Dis. 24, 606–613.

Aoyama, M., Kotani, J., and Usami, M. (2010). Butyrate and propionate induced activated or non-activated neutrophil apoptosis via HDAC inhibitor activity but without activating GPR-41/GPR-43 pathways. Nutrition 26, 653–661.

Besten, G. den, Eunen, K. van, Groen, A.K., Venema, K., Reijngoud, D.-J., Bakker, B.M., den Besten, G., van Eunen, K., Groen, A.K., Venema, K., et al. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J. Lipid Res. 54, 2325–2340.

Bjorklund, M.M., Hollensen, A.K., Hagensen, M.K., Dagnaes-Hansen, F., Christoffersen, C., Mikkelsen, J.G., and Bentzon, J.F. (2014). Induction of Atherosclerosis in Mice and Hamsters Without Germline Genetic Engineering. Circ. Res. 114, 1684–1689.

Brandsma, E., Houben, T., Fu, J., Shiri-Sverdlov, R., and Hofker, M.H. (2015). The immunity-diet-microbiota axis in the development of metabolic syndrome. Curr. Opin. Lipidol. 26, 73–81.

Cavaglieri, C.R., Nishiyama, A., Fernandes, L.C., Curi, R., Miles, E.A., and Calder, P.C. (2003). Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. Life Sci. 73, 1683–1690.

Chi, H., Messas, E., Levine, R.A., Graves, D.T., and Amar, S. (2004). Interleukin-1 Receptor Signaling Mediates Atherosclerosis Associated With Bacterial Exposure and/or a High-Fat Diet in a Murine Apolipoprotein E Heterozygote Model: Pharmacotherapeutic Implications. Circulation 110, 1678–1685.

Cox, M.A., Jackson, J., Stanton, M., Rojas-Triana, A., Bober, L., Laverty, M., Yang, X., Zhu, F., Liu, J., Wang, S., et al. (2009). Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E(2) and cytokines. World J. Gastroenterol. 15, 5549–5557.

DAWBER, T.R., MEADORS, G.F., MOORE, F.E., and Jr. (1951). Epidemiological approaches to heart disease: the Framingham Study. Am. J. Public Health Nations. Health 41, 279–281.

Drechsler, M., Megens, R.T.A., Van Zandvoort, M., Weber, C., and Soehnlein, O. (2010). Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. Circulation 122, 1837–1845.

Elhage, R., Maret, A., Pieraggi, M.T., Thiers, J.C., Arnal, J.F., and Bayard, F. (1998). Differential effects of interleukin-1 receptor antagonist and tumor necrosis factor binding protein on fatty-streak formation in apolipoprotein E-deficient mice. Circulation 97, 242–244.

Emoto, T., Yamashita, T., Sasaki, N., Hirota, Y., Hayashi, T., So, A., Kasahara, K., Yodoi, K., Matsumoto, T., Mizoguchi, T., et al. (2016). Analysis of Gut Microbiota in Coronary Artery Disease Patients: a Possible Link between Gut Microbiota and Coronary Artery Disease. J. Atheroscler. Thromb. 23, 908–921.

Endo, A., Kuroda, M., and Tanzawa, K. (1976). Competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme a reductase by ML-236A and ML-236B fungal metabolites, having hypocholesterolemic activity. FEBS Lett. 72, 323–326.

ENDO, A., KURODA, M., and TSUJITA, Y. (1976). ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogensis produced by Penicillium citrinum. J. Antibiot. (Tokyo). 29, 1346–1348.

Ference, B.A., Ginsberg, H.N., Graham, I., Ray, K.K., Packard, C.J., Bruckert, E., Hegele, R.A., Krauss, R.M., Raal, F.J., Schunkert, H., et al. (2017). Low-density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. Eur. Heart J. 38, 2459–2472.

Geva-Zatorsky, N., Sefik, E., Kua, L., Pasman, L., Tan, T.G., Ortiz-Lopez, A., Yanortsang, T.B., Yang, L., Jupp, R., Mathis, D., et al. (2017). Mining the Human Gut Microbiota for Immunomodulatory Organisms. Cell 168, 928–943.e11.
Gofman, J.W., Delalla, O., Glazier, F., Freeman, N.K., Lindgren, F.T., Nichols, A. V., Strisower, B., and Tamplin, A.R. (2007). The serum lipoprotein transport system in health, metabolic disorders, atherosclerosis and coronary heart disease. J. Clin. Lipidol. 1, 104–141.

Group, S.S.S.S. (1994). Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet (London, England) 344, 1383–1389.

Hayashi, C., Viereck, J., Hua, N., Phinikaridou, A., Madrigal, A.G., Gibson, F.C., Hamilton, J.A., and Genco, C.A. (2011). Porphyromonas gingivalis accelerates inflammatory atherosclerosis in the innominate artery of ApoE deficient mice. Atherosclerosis 215, 52–59.

Jie, Z., Xia, H., Zhong, S.-L., Feng, Q., Li, S., and Liang, S. (2017). The gut microbiome in atherosclerotic cardiovascular disease. Nat. Commun. 1–11.

Karlsson, F.H., Fåk, F., Nookaew, I., Tremaroli, V., Fagerberg, B., Petranovic, D., Bäckhed, F., and Nielsen, J. (2012). Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat. Commun. 3, 1245.

Khosravi, A., Yáñez, A., Price, J.G., Chow, A., Merad, M., Goodridge, H.S., and Mazmanian, S.K. (2014). Gut microbiota promote hematopoiesis to control bacterial infection. Cell Host Microbe 15, 374–381.

Kirii, H., Niwa, T., Yamada, Y., Wada, H., Saito, K., Iwakura, Y., Asano, M., Moriwaki, H., and Seishima, M. (2003). Lack of Interleukin-1beta Decreases the Severity of Atherosclerosis in ApoE-Deficient Mice. Arterioscler. Thromb. Vasc. Biol. 23, 656–660.

Koeth, R.A., Wang, Z., Levison, B.S., Buffa, J.A., Org, E., Sheehy, B.T., Britt, E.B., Fu, X., Wu, Y., Li, L., et al. (2013). Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat. Med. 19, 576–585.

Koren, O., Spor, A., Felin, J., Fåk, F., Stombaugh, J., Tremaroli, V., Behre, C.J., Knight, R., Fagerberg, B., Ley, R.E., et al. (2011). Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc. Natl. Acad. Sci. U. S. A. 108 Suppl 1, 4592–4598.

Li, J., Lin, S., Vanhoutte, P.M., Woo, C.W., and Xu, A. (2016). Akkermansia muciniphila protects against atherosclerosis by preventing metabolic endotoxemia-induced inflammation in Apoe-/- Mice. Circulation 133, 2434–2446.

Libby, P., Ridker, P.M., and Hansson, G.K. (2011). Progress and challenges in translating the biology of atherosclerosis. Nature 473, 317–325.
Matsuoka, K., and Kanai, T. (2015). The gut microbiota and inflammatory bowel disease. Semin. Immunopathol. 37, 47–55.

Moss, J.W., and Ramji, D.P. (2016). Cytokines: roles in atherosclerosis disease progression and potential therapeutic targets. Future Med. Chem. 8, 1317–1330.

Murphy, A.J., Akhtari, M., Tolani, S., Pagler, T., Bijl, N., Kuo, C.-L., Wang, M., Sanson, M., Abramowicz, S., Welch, C., et al. (2011). ApoE regulates hematopoietic stem cell proliferation, monocytosis, and monocyte accumulation in atherosclerotic lesions in mice. J. Clin. Invest. 121, 4138–4149.

Radi, Z.A., Kehrli, M.E., and Ackermann, M.R. (2001). Cell Adhesion Molecules, Leukocyte Trafficking, and Strategies to Reduce Leukocyte Infiltration. J. Vet. Intern. Med. 15, 516–529.

Ramji, D.P., and Davies, T.S. (2015). Cytokines in atherosclerosis: Key players in all stages of disease and promising therapeutic targets. Cytokine Growth Factor Rev. 26, 673–685.

Ridker, P.M., Everett, B.M., Thuren, T., MacFadyen, J.G., Chang, W.H., Ballantyne, C., Fonseca, F., Nicolau, J., Koenig, W., Anker, S.D., et al. (2017). Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N. Engl. J. Med. 377:1119-1131.

Sabatine, M.S., Giugliano, R.P., Keech, A.C., Honarpour, N., Wiviott, S.D., Murphy, S.A., Kuder, J.F., Wang, H., Liu, T., Wasserman, S.M., et al. (2017). Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. N. Engl. J. Med. 376, 1713–1722.

Sahyoun, N.R., Jacques, P.F., Zhang, X.L., Juan, W., and McKeown, N.M. (2006). Whole-grain intake is inversely associated with the metabolic syndrome and mortality in older adults. Am. J. Clin. Nutr. 83, 124–131.

Schirmer, M., Smeekens, S.P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E.A., Jansen, T., Jacobs, L., Bonder, M.J., Kurilshikov, A., et al. (2016). Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. Cell 167, 1125–1136.e8.

Stancu, C., and Sima, A. Statins: mechanism of action and effects. J. Cell. Mol. Med. 5, 378–387.

Swirski, F.K., and Nahrendorf, M. (2013). Leukocyte Behavior in Atherosclerosis, Myocardial Infarction, and Heart Failure. Science 339, 161–166.

Tang, W.H.W., and Hazen, S.L. (2014). The contributory role of gut microbiota in cardiovascular disease. J. Clin. Invest. 124, 4204–4211.

Tedelind, S., Westberg, F., Kjerrulf, M., and Vidal, A. (2007). Anti-inflammatory properties of the short-chain fatty acids acetate and propionate : A study with relevance to inflammatory bowel disease. 13, 2826–2832.

Yvan-Charvet, L., Pagler, T., Gautier, E.L., Avagyan, S., Siry, R.L., Han, S., Welch, C.L., Wang, N., Randolph, G.J., Snoeck, H.W., et al. (2010). ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation. Science 328, 1689–1693

Zhang, T., Kurita-Ochiai, T., Hashizume, T., Du, Y., Oguchi, S., and Yamamoto, M. (2010). Aggregatibacter actinomycetemcomitans accelerates atherosclerosis with an increase in atherogenic factors in spontaneously hyperlipidemic mice. FEMS Immunol. Med. Microbiol. 59, 143–151.

CHAPTER 2

The immunity-diet-microbiota axis in the development of metabolic syndrome

Eelke Brandsma[1], *Tom Houben*[2], *Jingyuan Fu*[3], *Ronit Shiri-Sverdlov*[2], *and Marten Hofker*[1]

1 Department of Pediatrics, Molecular Genetics, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
2 Department of Molecular Genetics, Maastricht University, Maastricht, The Netherlands
3 Department of Genetics, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Curr Opin Lipidol. 2015 Apr;26(2):73-81

Abstract

Purpose of the review

Recent evidence demonstrates that the gut-microbiota can be considered as one of the major factors causing metabolic and cardiovascular diseases.

Recent findings

Pattern recognition receptors as well as antimicrobial peptides are a key factor in controlling the intestinal microbiota composition. Deficiencies in these genes lead to changes in the composition of the gut-microbiota, causing leakage of endotoxins into the circulation, and the development of low-grade chronic inflammation and insulin resistance. Dietary composition can also affect the microbiota: a diet rich in saturated fats allows the expansion of pathobionts that damage the intestinal epithelial cell layer and compromise its barrier function. In contrast, a diet high in fiber supports the microbiota to produce short chain fatty acids, thereby promoting energy expenditure and protecting against inflammation and insulin resistance.

Summary

The interactions between the microbiota, innate immunity and diet play an important role in controlling metabolic homeostasis. A properly functioning innate immune system, combined with a low fat and high fiber diet, is important in preventing dysbiosis and reducing susceptibility to developing the metabolic syndrome and its associated cardiovascular diseases.

Introduction

Recent evidence points to an important role for the microbiota in the development of the metabolic syndrome. The link between the microbiota and host metabolism was initially discovered by Jeffrey Gordon's group, which showed that germfree mice are not susceptible to developing diet-induced obesity[1]. Conventionalization of germfree mice by the microbiota of lean mice led to an increase in adiposity and, strikingly, this effect was significantly stronger if the microbiota were derived from obese mice, demonstrating a causal role of microbiota composition on energy metabolism, and eventually leading to obesity. Microbiota analysis has shown that obese mice have a higher ratio of the firmicutes:bacteroidetes phyla and this composition has a greater capacity to harvest energy from the diet[2]. This means that the composition of the microbiota changes during obesity and that this change also contributes to the further development of obesity.

To study whether the microbiota composition also affects host metabolism in man, Ridaura et al performed an elegant study in which they transplanted the gut microbiota of human twins discordant for obesity into germfree mice. The mice that received microbiota from obese human subjects had a significantly increased body mass and adiposity compared to mice receiving microbiota from lean subjects[3], supporting that the microbiota contribute to development of obesity. Differences in microbiota composition and function have also been linked to the more advanced stages of metabolic syndrome, such as type 2 diabetes (T2D), non-alcoholic fatty liver disease (NAFLD), and cardiovascular diseases (CVD) [3,4**,5,6]. A change in microbiota composition is not merely associated with disease, but also appears to play a causal role in the development of the metabolic syndrome in humans, since transplanting the microbiota of healthy individuals into subjects diagnosed with metabolic syndrome led to an increased insulin sensitivity, thereby improving the metabolic phenotype of these patients[7]. Thus, the microbiota are an important factor in the development of the metabolic syndrome, although we are only just starting to understand how the microbiota influences the development of metabolic diseases. Microbiota composition is controlled by the mucosal immune system and is strongly influenced by dietary consumption. If the microbiota is not well maintained, this can lead to leakage of bacterial cell wall components into the circulation, which can

contribute to low-grade systemic inflammation, and consequently to metabolic syndrome. The microbiota can also influence the development of metabolic syndrome through the production of metabolites, such as short chain fatty acids, which can directly affect the host metabolism. In this review we will discuss the importance of the microbiota in the development of metabolic syndrome and how microbiota composition and its function is controlled by the mucosal immune system and affected by dietary intake.

Microbial control by innate immunity

The mucosal immune system is delicately balanced between tolerating commensal bacteria and eliminating pathogens. The first line of defense in the mucosal immune system is the physical barrier, consisting of epithelial cells kept together by tight junctions and the mucus layer lying on top of the epithelial cells. Goblet cells produce the mucus overlying the epithelial cells, physically separating the bacteria from the intestinal epithelial layer. The Paneth cells excrete antimicrobial peptides such as RegIIIy and alpha-defensins into this mucus layer. These peptides are important in preventing bacteria that are present naturally in the gut lumen from penetrating the mucus layer and adhering to the intestinal cell wall (Fig. 1).

Antimicrobial peptides

It has recently been shown that REGIIIy-/- mice have increased numbers of mucosa-associated-bacteria, which leads to the development of low-grade intestinal inflammation[8*,9]. RegIIIy is secreted by epithelial and Paneth cells under stimulation of the cytokine IL-22, thus IL-22 plays an important role in protecting the mucosal intestinal barrier (Fig. 1). IL-22R knockout mice show aberrant mucosal immunity and develop metabolic syndrome, characterized by an increase in bodyweight, hyperglycemia, insulin resistance, and decreased glucose tolerance. Strikingly, on administration of IL-22-Fc, which stimulates IL-22R, mice on a high fat diet (HFD) show reduced bodyweight, smaller fat pads, and better insulin sensitivity and glucose tolerance. Thus, mucosal protection by IL-22 plays an important role in protecting the host against metabolic syndrome[10**]. Protection by IL-22 can likely be explained by the reduced infiltration of bacteria in the intestinal epithelial cell layer, thus preventing low-

grade inflammation of the intestine and maintaining intestinal integrity. Maintenance of intestinal integrity is important to prevent translocation of bacteria or endotoxins from the gut lumen into the systemic circulation. Translocation of endotoxins (metabolic endotoxemia) into the systemic circulation leads to weight gain, higher insulin resistance and lower glucose tolerance[11]. Endotoxemia has also long been associated with the development of atherosclerosis in humans and increased production of endotoxins has been associated with symptomatic atherosclerosis in humans[5]. Altogether, leakage of endotoxins from the gut lumen into the systemic circulation contributes to the development of metabolic syndrome and atherosclerosis (Fig. 1).

In conclusion, a reduced control of microbiota localization by antimicrobial peptides could lead to more leakage of endotoxins into the circulation leading to inflammation in the liver, adipose tissue, and arteries.

Pattern recognition receptors

In addition to the physical features of the immune system preventing the translocation of bacteria, pattern recognition receptors (PRR) play an important role in protecting the human body from bacterial invasion by recognizing pathogen-associated molecular patterns (PAMPS), such as endotoxins. There are two major forms of PRRs: extracellular PRRs mainly consisting of the Toll-like receptor family (TLR) and intracellular PRRs, such as NOD-like receptors. Both types of PRRs have been shown to be important in controlling the microbiota composition and can thus affect the development of metabolic syndrome. TLRs as well as NOD-like receptors are expressed in the intestinal epithelial cells and are important for their proliferation, IgA production, tight junction formation, and the production of the antimicrobial peptides discussed earlier. This means the expression of PRRs in intestinal epithelial cells can play an important role in preventing a leaky gut[12,13]. PRRs are also abundantly expressed on innate immune cells, such as macrophages and dendritic cells, where they trigger phagocytosis of bacteria and the expression of cytokines and co-stimulatory molecules, which trigger the adaptive immunity[14]. Thus, PRRs in the intestine are important in controlling the gut microbiota and preventing translocation of bacteria into the systemic circulation.

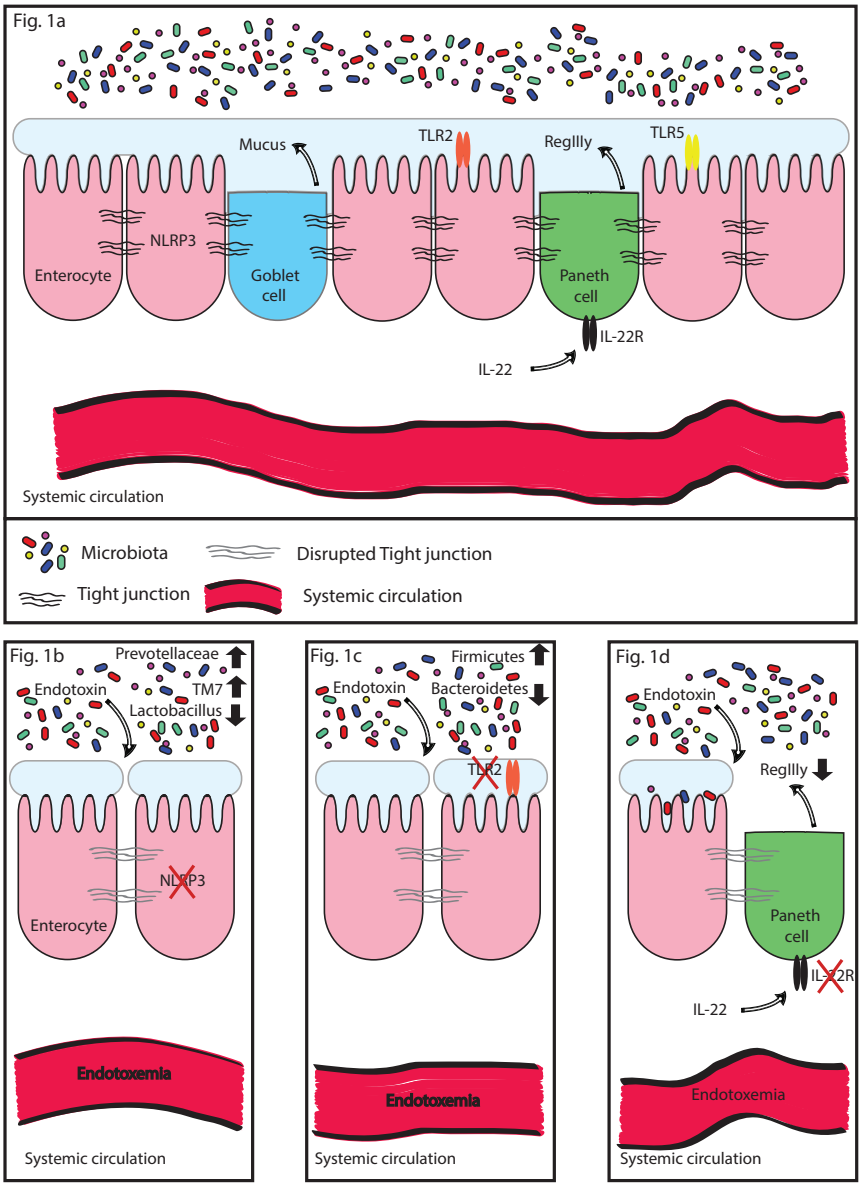


Figure 1. Microbiota control by immunity is essential to prevent dysbiosis and endotoxemia

Extracellular pattern recognition receptors

Germfree TLR2 knockout mice have been shown to be resistant to diet-induced insulin resistance, but they show a more severe development of metabolic syndrome upon colonization with microbiota. Their microbiota composition is changed showing a three-fold increase in firmicutes and a decrease in bacteroidetes. This change in composition leads to increased translocation of PAMPs, such as lipopolysaccharide into the circulation, causing subclinical inflammation and eventually insulin resistance, glucose intolerance, and obesity (Fig. 1). Strikingly, the metabolic phenotype is transferable to wild-type mice, by microbiota transfer, demonstrating that a lack of microbiota control is responsible for the observed phenotype[15]. Lack of microbial control in TLR5 knockout mice also leads to the metabolic syndrome developing under the influence of a HFD[16]. There are thus several lines of evidence showing that TLRs play an important role in controlling the microbiota composition and preventing metabolic diseases.

1a) The epithelial lining of the intestine is mainly formed by enterocytes, which are linked together by tight junctions. Goblet cells produce the mucus layer physically separating the microbiota, whereas Paneth cells excrete antimicrobial peptides such as RegIIIy into this layer upon stimulation of the IL22 receptor by IL-22. This prevents bacteria from the microbiota to infiltrate the intestinal epithelial cell layer. Intracellular and extracellular PRRs recognize endotoxins, binding of endotoxins to PRRs on enterocytes stimulates production of antimicrobial peptides and formation of tight junctions. 1b) The intracellular PRR NLRP3 plays an important role in shaping the microbiota composition. Disruption of NLRP3 function leads to dysbiosis, characterized by increased abundance of prevotellaceae and TM7, whereas abundance of Lactobacillus is decreased. 1c) Also extracellular PRRs are important in controlling microbiota composition. Disruption of TLR2 leads to dysbiosis, characterized by an increased firmicutes:bacteroidetes ratio. 1d) Disruption of stimulation of the IL22 receptor by Il-22 leads to decreased production of RegIIIy. Consequently bacteria from the microbiota can infiltrate the intestinal epithelial cell layer. Disturbance of NLRP3, TLR2 or IL22R function, leads to increased permeability due to disrupted tight junctions. Consequently endotoxins can leak through the intestinal epithelial cell layer into the systemic circulation, causing endotoxemia and contributing to low-grade systemic inflammation, which leads to development of the metabolic syndrome

NOD-like receptors

Inflammasomes belong to the NOD-like receptor (NLR) family and are important intracellular PRRs that play an important role in mucosal defense. Upon recognition of danger signals by an NLR (e.g. NLRP3), a multiprotein complex is formed with ASC and caspase 1, and together these are named inflammasomes. On formation of the inflammasome, caspase 1 is activated and pro-IL-1B and pro-IL-18 are post-translationally processed into their active isoforms IL-1B and IL-18. Disruption of the NLRP3 inflammasome function has been shown to influence the development of NAFLD.

The NLRP3 inflammasome influences the microbiota composition as well as the localization of the microbiota. NLRP3-/- mice have increased numbers of prevotellaceae and the bacterial phylum TM7, while having a reduced abundance of the lactobacillus genus (Fig. 1)[4**]. This change in microbiota composition leads to an increased severity of NAFLD under the influence of a methionine-choline-deficient diet. Increased severity of NAFLD is triggered by translocation of endotoxins into the systemic circulation, which activate TLR2 and TLR9 in the liver and consequently upregulate TNF-α and increase inflammation in the liver. Increased development of NAFLD depends on the inability of NLRP3-/- mice to process pro-IL-18 into IL-18, since IL-18-/- mice also showed a more severe development of NAFLD, which could be transferred to wild-type mice by microbiota transplantation[4**]. It is possible that the more severe development of NAFLD due to the translocation of endotoxins into the systemic circulation could be explained by the protective function that IL-18 exhibits against mucosal challenges or disruption of the intestinal mucosa[17].

NLRP3 does not merely play a role in controlling the microbiota composition but its expression in the intestinal epithelial cells is also important in preventing infiltration of the mucosal barrier by bacteria from the microbiota[13].

In conclusion, intracellular PRRs could have an important function in the protection against NAFLD, as highlighted by the function of NLRP3 in controlling the microbiota composition and localization in the intestine. Consequently, NLRP3 acts in preventing translocation of bacteria and endotoxins that can lead to low-grade systemic inflammation, which contributes to NAFLD development. Whether other intracellular PRRs also

have a protective role against NAFLD and whether they are also important for protection against T2D and CVD still needs to be established.

Diet-microbiota interactions in the development of metabolic syndrome

In addition to mucosal immunity, the diet strongly influences microbiota composition and function[18,19*]. Two general changes can be observed on consumption of a high-fat, low-fiber diet. First, pathobionts can expand during consumption of a HFD, and second, the abundance of protective bacteria, such as producers of short chain fatty acids, declines[19*,20,21**] (discussed later).

Pathobiont expansion under influence of dietary components

Decreased protection against pathobionts during consumption of a HFD is exemplified by the increased infiltration of adherent-invasive Escherichia Coli into the intestinal epithelial cell layer. This infiltration leads to less mucus thickness, and more intestinal inflammation and permeability (Fig. 2)[22]. Furthermore, expansion of pathobionts has been shown in humans with a diet rich in animal fats leading to expansion of the sulfate-reducing bacterium Bilophilia Wadsworthia (Fig. 2)[19*,21**]. Increased abundance of Bilophilia Wadsworthia in the microbiota has been associated with overall adiposity, dyslipidemia[6], and T2D and it triggers intestinal inflammation[21**,23]. This inflammation could possibly be explained by the production of H2S by Bilophilia Wadsworthia, which is toxic to host cells[24]. In addition, H2S deprives the enterocytes of energy by inhibiting oxidation of the most abundant energy source, butyrate[25]. Hence, increased infiltration of the intestinal epithelial layer, or production of toxic metabolites by pathobionts, can damage the intestinal epithelial layer and provide yet another cause of translocation of endotoxins or bacteria from the gut lumen into the systemic circulation, thereby contributing to the metabolic syndrome.

Modulation of dietary-derived choline by the microbiota promotes CVD

Modulation of dietary-derived choline, phosphatidylcholine or L-carnitine by the microbiota directly contributes to development of CVD. The

microbiota converts these dietary components into tri-methyl-amine (TMA) by the CutC gene cluster present in the microbiota[26,27]. TMA is taken up by the host and metabolized into tri-methylamine-N-oxide (TMAO) by flavin mono-oxygenase 3 in the liver. Increased levels of TMAO are associated with major cardiovascular events in humans and a causal role was demonstrated by promoting atherosclerosis in mice upon ingestion of TMAO[26-29**]. Altogether, conversion of dietary components by the gut microbiota leads to production of TMAO, which then promotes CVD.

Function of short chain fatty acids on host metabolism

The beneficial effects of "healthy" fiber-rich diets, which influence microbiota composition, are now better understood[30,31]. The microbiota of children consuming a high fiber diet have a lower firmicutes:bacteroidetes ratio. Furthermore the microbiota has an increased capacity to degrade dietary fibers, leading to the production of short chain fatty acids (SCFA), such as acetate, propionate and butyrate[30]. Human studies show a protective role for SCFA against metabolic syndrome, T2D and atherosclerosis[5,6,7].

The capacity of the microbiota to produce butyrate is negatively correlated with C-reactive protein levels in patients with atherosclerosis[5]. In addition, a decrease in butyrate production was found in T2D patients[6]. Causality for butyrate having a protective role against the development of metabolic diseases was demonstrated in a mouse study that showed that administering butyrate to mice on a HFD prevented the development of obesity and insulin resistance[32].

The mode of action for the protective role of SCFA against metabolic syndrome is mainly through binding to GPR41 and GPR43. These G-coupled receptors are widely expressed, including in tissues with an immunological function such as the spleen, lymph nodes, and bone marrow, as well as in metabolic tissues such as the large intestine, adipose tissue, and pancreas[33-36]. Binding of propionate to GPR41 in sympathetic neurons leads to an increased heart rate and oxygen consumption, causing higher energy expenditure (Fig. 3)[37]. Energy uptake is also modulated by SCFA. The SCFA propionate and butyrate stimulate intestinal gluconeogenesis and has beneficial metabolic effects, including the prevention of obesity and improved glucose and insulin tolerance. Butyrate directly stimulates intestinal gluconeogenesis, whereas the effect of propionate

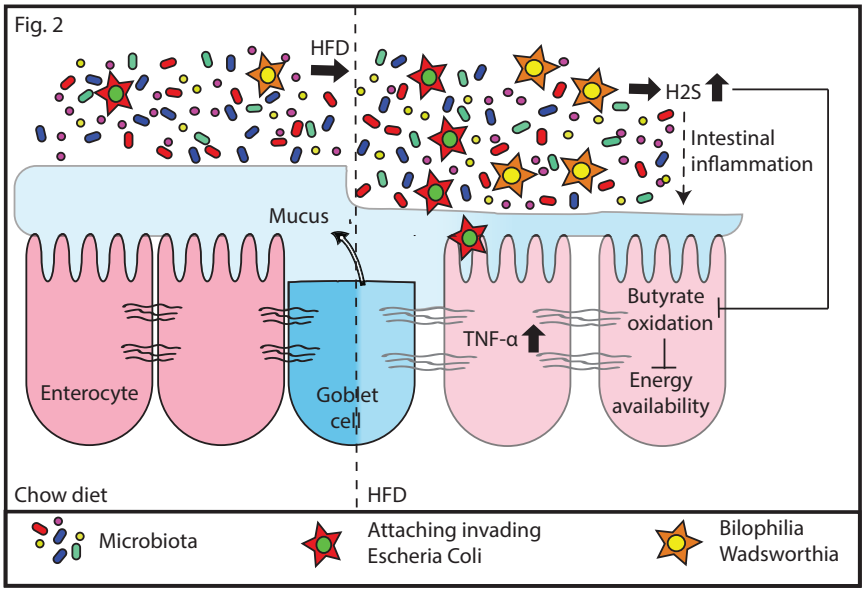


Figure 2. High fat diet induces pathobiont expansion

The intestinal epithelial layer protects against invasion of bacteria or endotoxins into the systemic circulation during consumption of a chow diet (left hand side of the figure). Consumption of a HFD leads to expansion of the pathobionts AIEC and Bilophilia Wadsworthia. AIEC infiltrate the intestinal epithelial layer, causing decreased mucus thickness, inflammation and increased permeability. Expansion of Bilophilia Wadsworthia leads to increased production of H2S. H2S is toxic to host cells and can possibly cause intestinal inflammation (dotted arrow). H2S can disturb the intestinal epithelial cell layer also by inhibiting the oxidation of butyrate by enterocytes, leading to decreased energy availability (right hand side of the figure).

is dependent on gut-brain neural signaling via GPR41[38*]. Furthermore, SCFA stimulate adipocytes to produce leptin, which is known to improve insulin sensitivity as well as to induce satiety, thereby controlling energy intake as well as uptake[39,40]. Moreover, SCFA also modulate glucose metabolism by stimulating L-cells in the intestine via GPR43, leading to the production of glucagon-like peptide 1 (GLP-1) and thereby improving glucose tolerance (Fig. 3)[41]. The importance of GPR43 in the signaling of SCFA was elegantly demonstrated by Kimura et al, who showed that GPR43-/- mice developed increased obesity, adiposity, white adipose

tissue inflammation, and decreased glucose tolerance[42**]. Whereas overexpression of GPR43 restricted to the adipose tissue suppresses insulin signaling in adipocytes, thereby preventing fat accumulation and metabolic dysfunction[42**]. In conclusion, SCFA are protective against metabolic syndrome by their modulation of the host metabolism via GPR41 and GPR43.

Bile acid modulation

A well-established role for the gut microbiota is the metabolism of bile acids [see review by Jones et al][43]. Primary bile salts are deconjugated by bile salt hydrolases, which are present in multiple gram-positive bacteria[44]. Recently the physiological role of bile salt hydrolase (BSH) by bacteria was demonstrated: the expression of bacterial BSH was found to reduce weight gain, and lower plasma cholesterol and hepatic triglycerides levels[45]. In addition, the microbiota affect the composition of the bile acid pool by 7-α/7-β dehydroxylation, desulfation and dehydrogenation, leading to the formation of secondary bile acids, such as deoxycholic acid, lithocholic acid, and tauro-beta-muricholic acid[43]. Bile acids have been shown to be important regulators of metabolic signaling via the Farnesoid X receptor (FXR) and the G-coupled protein receptor, TGR5 [46]. Stimulation of FXR improves the glucose and lipid metabolism, leading to increased insulin sensitivity and decreased steatosis in the liver. Activation of TGR5 in the intestine by bile acids leads to secretion of GLP-1, thereby improving glucose tolerance and insulin sensitivity. FXR is activated by bile acids with the following potency: chenodeoxycholic acid > deoxycholic acid > litohocholic acid >> cholic acid, whereas TGR5 is activated as follows: litohocholic acid > deoxycholic acid > chenodeoxycholic acid > cholic acid[46]. Next to the agonistic effect of bile acids on FXR, Tauro β-muricholate (TβMCA) has recently been reported to be an antagonist of FXR. In mice the microbiota reduces TMCA levels, leading to increased activation of FXR in the intestine and excretion of FGF15, which is transported to the liver and inhibits bile acids synthesis[47]. Although the potency of multiple bile acids in stimulating FXR and TGR5 are known, it is not yet fully understood how changes in the composition of the bile acid pool affect the metabolism. It should be noted that bile acid metabolism differs considerably between mice and humans. The human bile acid pool is much more hydrophobic. Hence,

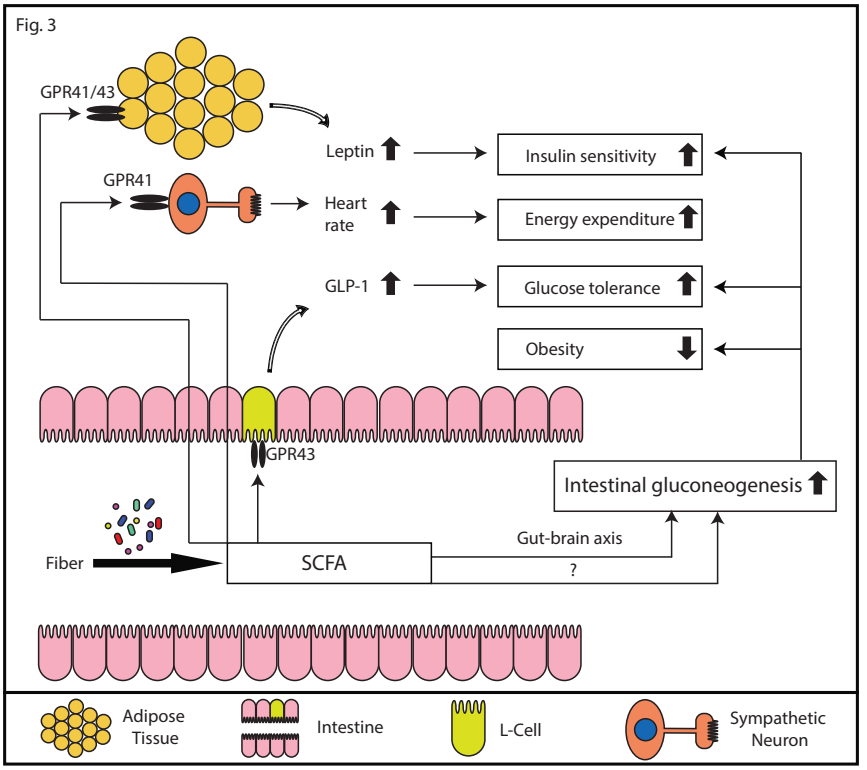


Figure 3. SCFA are protective against development of metabolic syndrome

Fibers are converted by the microbiota to SCFA. Production of SCFA stimulates L-cells in the intestine via GPR43 to produce GLP-1, this leads to increased glucose tolerance. In addition, SCFA can directly stimulate intestinal gluconeogenesis (IGN), either by direct stimulation of IGN via an unknown mechanism or by stimulation of IGN dependent on the gut-brain axis. Increased IGN leads to decreased obesity, increased glucose tolerance and insulin sensitivity. Next to protective effects in the intestine, SCFA are also taken up into the circulation where stimulation of GPR41 or GPR43 in adipose tissue leads to increased production of leptin and thereby increased insulin sensitivity. Furthermore SCFA can also stimulate sympathetic neurons leading to an increased heart rate and consequently increased energy expenditure.

data for mice cannot be translated easily to the human situation. Yet, both in humans and mice the microbiota are important for regulating the bile acid composition, and can thereby regulate the metabolism via FXR and TGR5. Which members of the bacterial community are responsible for the

many different modifications of bile acids, as well as the effect of the bile acid pool composition on metabolism, is not yet well understood and this would be an interesting target for future research.

Conclusion

There is no doubt that the intestinal microbiota plays an important role in host metabolism and that dysbiosis is a strong risk factor in the development of metabolic syndrome and CVD. We are just starting to understand how the host's immune system and dietary intake can influence the microbiota composition and function. Technological advances include lower sequencing costs, which enables the generation of meta-genomic data to identify individual species and specific functions, such as the presence of the CutC gene cluster. The strain-specific information is crucial to gaining a better understanding of the complex interactions between the different members in the microbiota and their cross-talk with the host in relation to metabolic dysfunction. This could lead to the identification of new biomarkers for metabolic and other diseases. There is furthermore an urgent need for meta-genomic data from prospective cohort studies that include multiple layers of information, such as dietary intake and metabolic data (stool, plasma). The causal roles of newly identified pathobionts or the functions of specific members of the microbiota found in mice or human studies need to be validated in germfree and gnotobiotic mouse models, as well as in human intervention studies with individual bacterial strains. Ultimately, better insight into the immunity-diet-microbiota axis is expected to reveal new targets for intervention and an enormous potential for the prevention of cardiovascular disease.

Key points

1) The mucosal immune system is essential for microbiota control and prevention of endotoxemia

2) Consumption of high fat diets leads to expansion of pathobionts damaging the intestinal epithelial layer

3) Production of SCFA by the microbiota is protective against development of the metabolic syndrome

Acknowledgments

We would like to thank Jackie Senior for editing the manuscript and Bert Groen for his advice.

Financial support and sponsorship

E.T. Brandsma and T. Houben are supported by the CVON IN-CONTROL grant [CVON2012-03]. R. Shiri-Sverdlov is supported by an NWO-VIDI grant 016.126.327 and J. Fu is supported by VIDI grant 864.13.013.

Conflicts of interest

None

References

Papers of particular interest, published within the annual period of review, have been highlighted as:
** of special interest*
*** of outstanding interest*

(1) Backhed F, Ding H, Wang T et al. The gut microbiota as an environmental factor that regulates fat storage. Proc.Natl.Acad.Sci.U.S.A. 2004;101:15718-15723.

(2) Turnbaugh PJ, Ley RE, Mahowald MA et al. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027-1031.

(3) Ridaura VK, Faith JJ, Rey FE et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science 2013;341:1241214.

***(4) Henao-Mejia J, Elinav E, Jin C et al. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 2012;482:179-185.*
This study elegantly shows that intracellular pattern recognition receptors are essential in preventing dysbiosis. Development of dysbiosis leads to leakage of endotoxins into the systemic circulation, contributing to inflammation in the liver and thereby promoting development of NAFLD.

(5) Karlsson FH, Fak F, Nookaew I et al. Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat.Commun. 2012;3:1245.

(6) Qin J, Li Y, Cai Z et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 2012;490:55-60.

(7) Vrieze A, Van Nood E, Holleman F et al. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in individuals with metabolic syndrome. Gastroenterology 2012;143:913-6.e7.

**(8) Loonen LM, Stolte EH, Jaklofsky MT et al. REG3gamma-deficient mice have altered mucus distribution and increased mucosal inflammatory responses to the microbiota and enteric pathogens in the ileum. Mucosal Immunol. 2014;7:939-947.*
This study shows that the antimicrobial peptide Reg3y is essential to protect against bacteria penetrating into the intestinal epithelial layer.

(9) Vaishnava S, Yamamoto M, Severson KM et al. The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science 2011;334:255-258.

**(10) Wang X, Ota N, Manzanillo P et al. Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes. Nature 2014;514:237-241.
This study shows the essential role of mucosal immunology in protection against metabolic disorders. Obese mice are more susceptible to infiltration of the intestinal epithelial layer by citrobacter rodentium and have a reduced production of IL22. IL22R-/- mice are more susceptible to developing the metabolic syndrome, whereas administration of IL22 to mice fed a high fat diet protects them against the metabolic syndrome.

(11) Cani PD, Amar J, Iglesias MA et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761-1772.

(12) Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat.Rev.Immunol. 2010;10:131-144.

(13) Song-Zhao GX, Srinivasan N, Pott J et al. Nlrp3 activation in the intestinal epithelium protects against a mucosal pathogen. Mucosal Immunol. 2014;7:763-774.

(14) Hemmi H, Akira S. TLR signalling and the function of dendritic cells. Chem. Immunol.Allergy 2005;86:120-135.

(15) Caricilli AM, Picardi PK, de Abreu LL et al. Gut microbiota is a key modulator of insulin resistance in TLR 2 knockout mice. PLoS Biol. 2011;9:e1001212.

(16) Vijay-Kumar M, Aitken JD, Carvalho FA et al. Metabolic syndrome and altered gut microbiota in mice lacking Toll-like receptor 5. Science 2010;328:228-231.

(17) Carvalho FA, Nalbantoglu I, Aitken JD et al. Cytosolic flagellin receptor NLRC4 protects mice against mucosal and systemic challenges. Mucosal Immunol. 2012;5:288-298.

(18) Cotillard A, Kennedy SP, Kong LC et al. Dietary intervention impact on gut microbial gene richness. Nature 2013;500:585-588.

*(19) David LA, Maurice CF, Carmody RN et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559-563.
This study shows that diet is a major factor influencing microbiota composition and that a diet low in dietary fibers and rich in animal fats leads to expansion of Bilophilia Wadsworthia.

(20) Le Chatelier E, Nielsen T, Qin J et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013;500:541-546.

**(21) Devkota S, Wang Y, Musch MW et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature 2012;487:104-108.
This study elegantly shows that a diet rich in animal fat triggers taurine conjugation of bile acids, triggering the expansion of the pathobiont Bilophilia Wadsworthia, and consequently promoting intestinal inflammation.

(22) Martinez-Medina M, Denizot J, Dreux N et al. Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 2014;63:116-124.

(23) Ding S, Chi MM, Scull BP et al. High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS One 2010;5:e12191.

(24) Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. Nat.Immunol. 2013;14:685-690.

(25) Medani M, Collins D, Docherty NG et al. Emerging Role of Hydrogen Sulfide in Colonic Physiology and Pathophysiology. Inflamm.Bowel Dis. 2011;17:1620-1625.

(26) Craciun S, Balskus EP. Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. Proc.Natl.Acad.Sci.U.S.A. 2012;109:21307-21312.

(27) Wang Z, Klipfell E, Bennett BJ et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57-63.

(28) Koeth RA, Wang Z, Levison BS et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat.Med. 2013;19:576-585.

**(29) Tang WHW, Wang Z, Levison BS et al. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. N.Engl.J.Med. 2013;368:1575-1584.
This study elegantly shows that conversion of phophatidylcholine to trimethylamine by the microbiota leads to increased TMAO production in humans. They correlate TMAO production with major cardiovascular events in a large human cohort study, thereby implying an important role for the microbiota in the development of cardiovascular disease in humans.

(30) De Filippo C, Cavalieri D, Di Paola M et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proc.Natl.Acad.Sci.U.S.A. 2010;107:14691-14696.

(31) Roberfroid MB. Inulin-type fructans: Functional food ingredients. J.Nutr. 2007;137:2493S-2502S.

(32) Gao Z, Yin J, Zhang J et al. Butyrate Improves Insulin Sensitivity and Increases Energy Expenditure in Mice. Diabetes 2009;58:1509-1517.

(33) Brown AJ, Goldsworthy SM, Barnes AA et al. The orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J.Biol.Chem. 2003;278:11312-11319.

(34) Nilsson NE, Kotarsky K, Owman C, Olde B. Identification of a free fatty acid receptor, FFA(2)R, expressed on leukocytes and activated by short-chain fatty acids. Biochem.Biophys.Res.Commun. 2003;303:1047-1052.

(35) Le Poul E, Loison C, Struyf S et al. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. J.Biol.Chem. 2003;278:25481-25489.

(36) Regard JB, Sato IT, Coughlin SR. Anatomical Profiling of G Protein-Coupled Receptor Expression. Cell 2008;135:561-571.

(37) Kimura I, Inoue D, Maeda T et al. Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). Proc.Natl.Acad.Sci.U.S.A. 2011;108:8030-8035.

**(38) De Vadder F, Kovatcheva-Datchary P, Goncalves D et al. Microbiota-Generated Metabolites Promote Metabolic Benefits via Gut-Brain Neural Circuits. Cell 2014;156:84-96.*
This study shows the importance of short chain fatty acids derived from microbiota in stimulating intestinal gluconeogenesis, thereby promoting metabolic benefits on body weight and glucose metabolism.

(39) Xiong YM, Miyamoto N, Shibata K et al. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc.Natl.Acad.Sci.U.S.A. 2004;101:1045-1050.

(40) Zaibi MS, Stocker CJ, O'Dowd J et al. Roles of GPR41 and GPR43 in leptin secretory responses of murine adipocytes to short chain fatty acids. FEBS Lett. 2010;584:2381-2386.

(41) Tolhurst G, Heffron H, Lam YS et al. Short-Chain Fatty Acids Stimulate Glucagon-Like Peptide-1 Secretion via the G-Protein-Coupled Receptor FFAR2. Diabetes 2012;61:364-371.

**(42) Kimura I, Ozawa K, Inoue D et al. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nature Communications 2013;4:1829.
This study shows the importance for signaling of short chain fatty acids via GPR43 in the protection against the metabolic syndrome. GPR43-/- mice develop obesity and decreased glucose tolerance, whereas overexpression of GPR43 in adipocytes protects against development of the metabolic syndrome by preventing insulin-mediated fat accumulation in the adipose tissue.

(43) Jones ML, Martoni CJ, Ganopolsky JG et al. The human microbiome and bile acid metabolism: dysbiosis, dysmetabolism, disease and intervention. Expert Opin.Biol.Ther. 2014;14:467-482.

(44) Schwarz M, Russell DW, Dietschy JM, Turley SD. Marked reduction in bile acid synthesis in cholesterol 7 alpha-hydroxylase-deficient mice does not lead to diminished tissue cholesterol turnover or to hypercholesterolemia. J.Lipid Res. 1998;39:1833-1843.

(45) Joyce SA, MacSharry J, Casey PG et al. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. Proc.Natl.Acad. Sci.U.S.A. 2014;111:7421-7426.

(46) de Aguiar Vallim TQ, Tarling EJ, Edwards PA. Pleiotropic roles of bile acids in metabolism. Cell.Metab. 2013;17:657-669.

(47) Sayin SI, Wahlstrom A, Felin J et al. Gut Microbiota Regulates Bile Acid Metabolism by Reducing the Levels of Tauro-beta-muricholic Acid, a Naturally Occurring FXR Antagonist. Cell Metabolism 2013;17:225-235.

CHAPTER 3

A Pro-Inflammatory Gut Microbiota Increases Systemic Inflammation and Accelerates Atherosclerosis

***Eelke Brandsma*[1]**, *MSc; Niels J. Kloosterhuis[1], BSc; Mirjam Koster[1,2], BSc; Daphne Dekker[1], BSc; Marion Gijbels[3,4], PhD; Saskia van der Velden[3], BSc; Melany Ríos Morales[1], MSc; Martijn J.R. van Faassen[5], MSc; Marco Loreti[1], BSc; Alain de Bruin[2], PhD; Jingyuan Fu[1,6], PhD; Folkert Kuipers[1,4], PhD; Barbara Bakker[1], PhD; Marit Westerterp[1], PhD; Menno de Winther[3,7], PhD; Marten H. Hofker[1]†, PhD; Bart van de Sluis[1]*, PhD and Debby P.Y. Koonen[1]*, PhD*

† Deceased
**Equal contribution*

1. University of Groningen, University Medical Center Groningen, Department of Pediatrics, Groningen, the Netherlands
2. Dutch Molecular Pathology Center, Department of Pathobiology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
3. Experimental Vascular Biology, Department of Medical Biochemistry, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands
4. Department of Pathology and Department of Molecular Genetics, CARIM, Maastricht University, Maastricht, the Netherlands
5. University of Groningen, University Medical Center Groningen, Department of Laboratory Medicine, Groningen, the Netherlands
6. University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, the Netherlands
7. Institute for Cardiovascular Prevention (IPEK), Ludwig Maximilian's University, Munich, Germany

Circ Res . 2019;124:94-100. DOI: 10.1161/CIRCRESAHA.118.313234.

Abstract

Rationale

Several studies have suggested a role for the gut microbiota in inflammation and atherogenesis. A causal relation relationship between gut microbiota, inflammation and atherosclerosis has not been explored previously.

Objective

Here, we investigated whether a pro-inflammatory microbiota from *Caspase1*$^{-/-}$ (*Casp1*$^{-/-}$) mice accelerates atherogenesis in *Ldlr*$^{-/-}$ mice.

Method and Results

We treated female *Ldlr*$^{-/-}$ mice with antibiotics and subsequently transplanted them with fecal microbiota from *Casp1*$^{-/-}$ mice based on a co-housing approach. Autologous transplantation of fecal microbiota of *Ldlr*$^{-/-}$ mice served as control. Mice were co-housed for 8 or 13 weeks and fed chow or a high-fat cholesterol-rich (HFC) diet. Fecal samples were collected, and factors related to inflammation, metabolism, intestinal health and atherosclerotic phenotypes were measured. Unweighted Unifrac distances of 16S rDNA sequences confirmed the introduction of the *Casp1*$^{-/-}$ and *Ldlr*$^{-/-}$ microbiota into *Ldlr*$^{-/-}$ mice (referred to as *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) or *Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*)* mice). Analysis of atherosclerotic lesion size in the aortic root demonstrated a significant 29% increase in plaque size in 13-week HFC-fed *Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*)* mice compared to *Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*)* mice. We found increased numbers of circulating monocytes and neutrophils and elevated pro-inflammatory cytokine levels in plasma in HFC-fed *Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*)* compared to *Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*)* mice. Neutrophil accumulation in the aortic root of *Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*)* mice was enhanced compared to *Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*)* mice. 16S-rDNA-encoding sequence analysis in feces identified a significant reduction in the short-chain fatty acid (SCFA)-producing taxonomies *Akkermansia, Christensenellaceae, Clostridium* and *Odoribacter* in *Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*)* mice. Consistent with these findings, cumulative concentrations of the anti-inflammatory SCFAs proprionate, acetate and butyrate in the cecum were significantly reduced in 13-week HFC-fed *Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*)* compared to *Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*)* mice.

Conclusion

Introduction of the pro-inflammatory *Casp1*$^{-/-}$ microbiota into *Ldlr*$^{-/-}$ mice enhances systemic inflammation and accelerates atherogenesis.

Introduction

Atherosclerosis, the main underlying cause of cardiovascular disease, is traditionally considered a lipid-driven disease. However, numerous studies have shown that atherosclerosis is influenced by the innate and adaptive immune system with cytokines involved in all stages of atherogenesis.[1,2] Moreover, the CANTOS-trial demonstrated that an antibody against interleukin-1β reduced recurrent cardiovascular events in patients with a previous myocardial infarction, indicating that inflammation enhances cardiovascular risk in humans.[3]

Gut microbiota is known to be involved in the shaping of the immune system during early life. Recent studies have suggested a role for the gut microbiota in the regulation of inflammation by influencing differentiation of inflammatory cell types, cytokine production and haematopoiesis.[4–6] A leaky gut and alterations in gut microbiota composition can both lead to leakage of endotoxins into the circulation that promote systemic inflammation and to the development of obesity and related metabolic diseases.[7,8] Symptomatic atherosclerosis is associated with an altered gut metagenome in the human population [9,10] and bacterial DNA has been detected in atherosclerotic plaques.[11] Furthermore, a high blood concentration of the microbiota-dependent metabolite trimethyl-amine-N-oxide (TMAO) has been linked to an increased risk of atherosclerosis,[12–14] indicating a pivotal role for the gut microbiota in atherogenesis. In addition, germ-free *ApoE*-deficient (*ApoE*$^{-/-}$) mice showed lower circulating lipopolysaccharide levels, reduced systemic inflammation, and decreased atherogenesis compared to conventionally raised *ApoE*$^{-/-}$ mice[15]. Taken together, these findings suggest a triangular relationship between the gut microbiota, host immunity and atherogenesis, however, proof to support a pro-inflammatory role for the gut microbiota in atherogenesis is lacking. To examine whether introduction of a pro-inflammatory gut microbiota accelerates atherogenesis, we exposed female *Ldlr*$^{-/-}$ mice to the pro-inflammatory gut microbiota of *Casp1*$^{-/-}$ mice,[7] as previous reports have demonstrated that alterations in their microbiota sensitize mice to the development of several inflammatory diseases.[7,16] The gut microbiota of *Casp1*$^{-/-}$ mice promoted atherosclerosis and increased blood leukocyte numbers, pro-inflammatory plasma cytokines, and neutrophil accumulation in atherosclerotic plaques, while plasma lipid and TMAO levels, and gut integrity were unaffected. The *Casp1*$^{-/-}$ microbiota reduced microbiota-

derived anti-inflammatory short-chain-fatty acids (SCFAs).

Methods

The methods section is available in the Online Data.

Results

Casp1$^{-/-}$ microbiota successfully introduced into *Ldlr*$^{-/-}$ mice

To study whether a pro-inflammatory microbiota accelerates atherogenesis, we exposed antibiotic-treated *Ldlr*$^{-/-}$ mice to the gut microbiota of *Casp1*$^{-/-}$ mice through fecal microbiota transplantation *via* a co-housing approach[7] (Figure 1A). Autologous transplantation of fecal microbiota from *Ldlr*$^{-/-}$ mice into antibiotic-treated *Ldlr*$^{-/-}$ mice *via* a co-housing approach[7] served as control. Analysis of fecal microbiota composition at time of sacrifice revealed both co-housing and diet-associated changes in gut microbial ecology (Figure 1B). Unweighted UniFrac distances of 16S rDNA sequences, a measure for b-diversity, demonstrated clustering between the *Ldlr*$^{-/-}$ mice receiving *Casp1*$^{-/-}$ microbiota (referred to as *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice) and the *Casp1*$^{-/-}$ donor mice (Figure 1B, Online Table II). Analogously, we observed clustering between *Ldlr*$^{-/-}$ mice receiving the autologous microbiota transplantation (referred to as *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) mice) and their respective donor mice. We also observed a clear separation between mice fed chow or a HFC diet (Figure 1B, Online Table I), and this was consistent for all donor and recipient mice. As expected, a-diversity was not different between *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) and *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice (Online Figure IB). Altogether, these data demonstrate that *Casp1*$^{-/-}$ and *Ldlr*$^{-/-}$ microbiota were successfully transferred into *Ldlr*$^{-/-}$ mice.

Casp1$^{-/-}$ dysbiosis promotes atherosclerosis in *Ldlr*$^{-/-}$ mice fed a HFC-diet

We analyzed atherosclerotic lesion size in the aortic root and we found that *Casp1*$^{-/-}$ microbiota did not affect atherosclerotic lesion size in *Ldlr*$^{-/-}$ mice fed chow or a HFC diet for 8 weeks (Figure 2A, B). However, atherosclerotic lesion size was increased by 29% in *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice compared to *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) mice following 13 weeks of HFC-feeding (Figure 2A, B; $p < 0.05$). The collagen and macrophage content in aortic root sections was not different between the mice (Online Figure IIA), indicating that lesion size but not severity was increased. In the aortic arches, gene

expression of several macrophage-related and inflammatory markers were similar between HFC-fed mice with the exception of a significant increase in *Il-10* expression in *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice (Online Figure IIB). Body weight, plasma triglyceride and cholesterol levels (Online Figure IIIA-D) also did not differ and no alteration was observed in plasma levels of TMAO, its TMA precursors (choline, L-carnitine, betaine, and g-butyrobetaine; Online Figure IIIE), and TMAO-producing taxonomies (Online Figure IIIF). Altogether, these results exclude plasma lipid levels and TMAO as factors that contribute to the increased atherosclerotic lesions in *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice.

Casp1$^{-/-}$ dysbiosis promotes inflammation

Next, we assessed whether *Casp1*$^{-/-}$ dysbiosis accelerates atherosclerosis by increasing plasma inflammatory cytokines. We found a significant elevation in the plasma levels of IL-1β, IL-2, IL-10 and IFN-γ in *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice after 13 weeks of HFC diet (Figure 3A), while CXCL1, TNF-α, IL-5 and IL-6 were not affected (Online Figure IVB). Flow cytometry analysis showed an increase in the number of blood Ly6C^{lo} and Ly6C^{hi} monocytes and neutrophils in *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice compared to *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) mice accompanied by an increase in white blood cell count (Figure 3B, Online Figure IVA), with leukocyte percentages being unchanged (Online Figure IVC). We also observed increased neutrophil accumulation in atherosclerotic plaques of *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice compared to *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) fed a HFC diet for 13 weeks (Figure 3C, D). These data suggest that *Casp1*$^{-/-}$ dysbiosis enhances IL-1β plasma levels resulting in monocytosis and neutrophilia and increased neutrophil accumulation in atherosclerotic plaques.

Exposure to Casp1$^{-/-}$ microbiota does not impair intestinal barrier function in Ldlr$^{-/-}$ mice fed a HFC diet

A disturbance in microbiota composition may affect intestinal integrity and subsequently promote systemic inflammation[7,8] To investigate the effect of *Casp1*$^{-/-}$ microbiota on intestinal barrier function, we analyzed the gut microbiota composition using LEfSe analysis of 16S rDNA encoding sequences. We identified 34 microbial taxonomies that differed in abundance between *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) and *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice (Online Figure VA). *Casp1*$^{-/-}$ dysbiosis resulted in a significant expansion of the

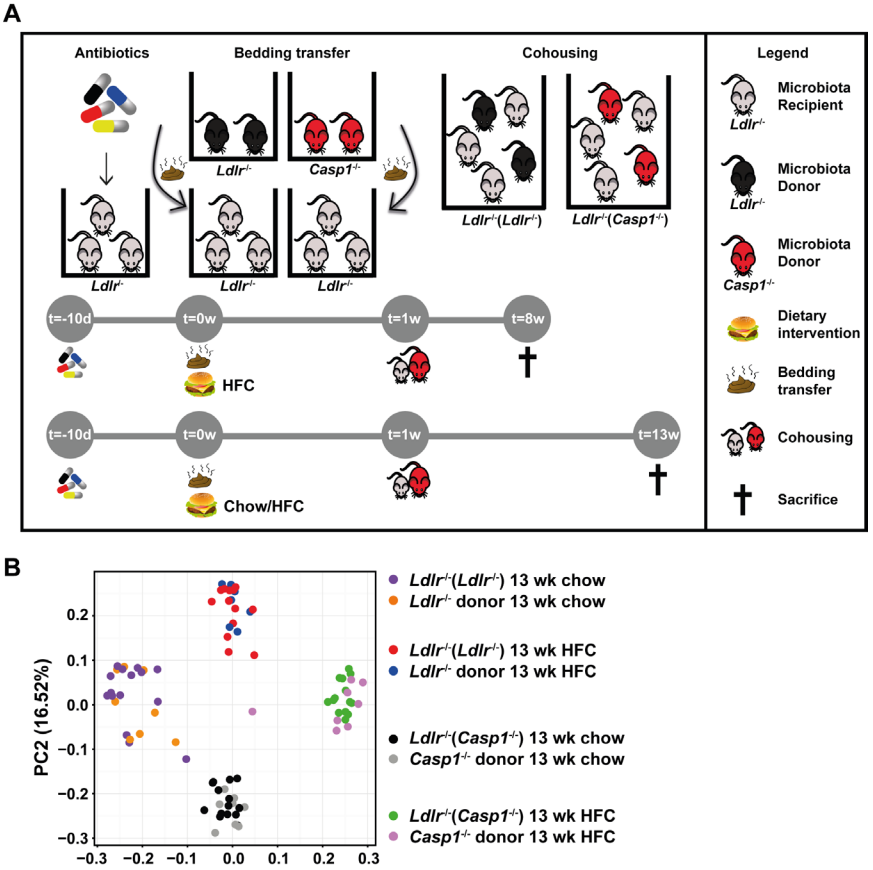


Figure 1 – Transplantation of *Casp1*$^{-/-}$ *microbiota into Ldlr*$^{-/-}$ mice *via* a co-housing approach. *Female Ldlr*$^{-/-}$ *mice aged 12 weeks were exposed to fecal microbiota derived from Casp1*$^{-/-}$ *or Ldlr*$^{-/-}$ *mice for 8 or 13 weeks while fed a chow diet or a high-fat cholesterol (HFC) diet. (***A***) Experimental setup of the co-housing approach. Female Ldlr*$^{-/-}$ *mice were orally gavaged with a cocktail of broad spectrum antibiotics for a period of 10 days to suppress intestinal microbes. This was followed by daily transfer of used bedding material from cages housing non-antibiotic-treated Ldlr*$^{-/-}$ *(donor) or Casp1*$^{-/-}$ *(donor) mice to cages housing the antibiotic-treated Ldlr*$^{-/-}$ *mice for 1 week. During this period the mice were kept on chow diet or switched to a HFC diet for the remainder of the study. The antibiotic-treated Ldlr*$^{-/-}$ *mice were then cohoused with non-antibiotic-treated Casp1*$^{-/-}$ *mice (referred to as Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*) mice) or Ldlr*$^{-/-}$ *mice (autologous transplantation, referred to as Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*) mice) in a 3:2 ratio for a period of 8 or 13 weeks. (***B***) Principal coordinate analysis plot of Unweighted UniFrac distance on the basis of*

genera *Bilophila, Streptococcus* and *Mucispirillum* (Online Figure VB-D) under both chow- and HFC-diet conditions. Although these genera are associated with intestinal inflammation, and are known to expand under inflammatory conditions,[17] we did not observe any differences in intestinal barrier function, e.g. inflammation and epithelial injury (Online Figure VIA-C). In addition, mucus layer thickness of the colon (Online Figure VID, E) and *Muc-2* expression (Online Figure VIF) in the colon were not altered between groups, suggesting that the integrity of the mucus layer of the colon was not different between *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) and *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice. Although intestinal permeability was significantly impaired by HFC-feeding, only *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice fed chow diet displayed increased permeability compared to *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) mice (Online Figure VIG). These results indicate that *Casp1*$^{-/-}$ microbiota does not change the intestinal barrier function under HFC-diet conditions and therefore cannot explain the increase in plasma inflammatory cytokines.

Exposure to Casp1$^{-/-}$ microbiota lowers SCFA-producing microbial taxonomies and cecum concentration of SCFAs

We observed a significant reduction in the abundance of the SCFA-producing taxonomies *Akkermansia* (Figure 4A), *Christensenellaceae* (Figure 4B), *Clostridium* (Figure 4C) and *Odoribacter* (Figure 4D) in *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice. As previous studies have shown that SCFAs reduce inflammation,[18,19] we measured the concentrations of acetate, propionate and butyrate in the cecum of the mice. Consistent with the lower abundance of SCFA-producing taxonomies, a significant reduction was observed in the cumulative levels of these SCFAs in *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice compared to *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) mice (Figure 4E) and this was mainly due to lower acetate levels in the *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) mice. Thus, it is conceivable that a reduction in the anti-inflammatory SCFAs may have contributed to the increased levels of inflammatory plasma cytokines of mice exposed to *Casp1*$^{-/-}$ microbiota.

16S-rDNA-encoding sequences in feces collected from chow- and HFC-fed Ldlr$^{-/-}$ *mice exposed to Casp1*$^{-/-}$ *or Ldlr*$^{-/-}$ *microbiota for 13 weeks.*
Chow: *Ldlr*$^{-/-}$ *mice (donor), n= 8; Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*) mice, n=15; Casp1*$^{-/-}$ *mice (donor), n=9; Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*) mice, n=14.* **HFC**: *Ldlr*$^{-/-}$ *mice (donor), n=7; Ldlr*$^{-/-}$*(Ldlr*$^{-/-}$*) mice, n=13; Casp1*$^{-/-}$ *mice (donor), n=8; Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*) mice, n=14.*

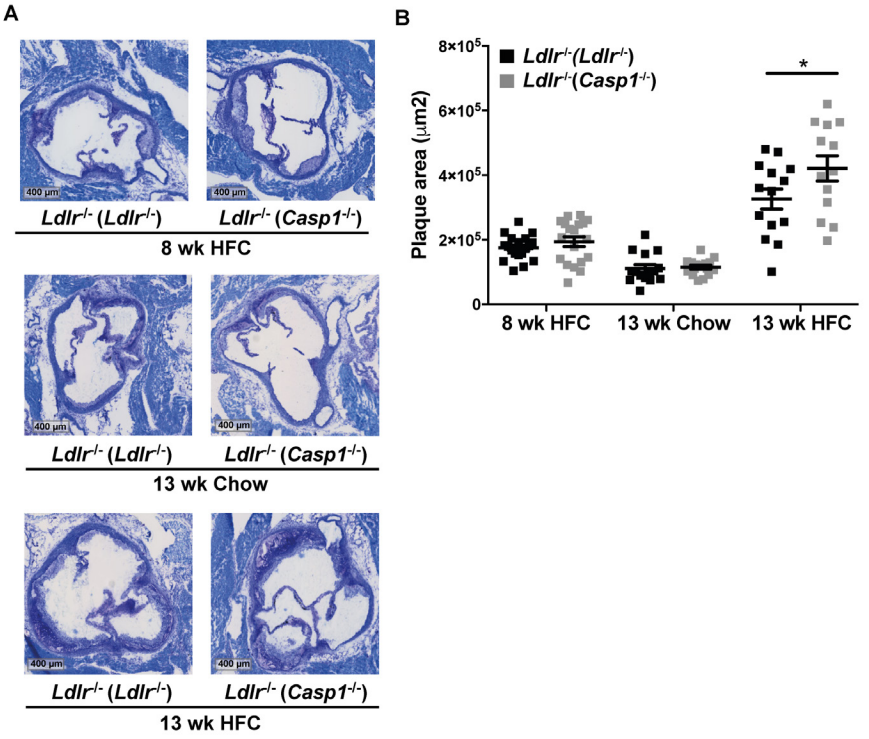


Figure 2 – Casp1$^{-/-}$ microbiota promotes atherosclerosis development in Ldlr$^{-/-}$ mice fed a HFC diet. (A) *Representative toluidine blue stained slides of the aortic root. Scale bars, 400 mm.* **(B)** *Quantification of atherosclerotic root lesion area. Chow (13 weeks): Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=15; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=16. HFC (8 weeks): Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=19; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=19. HFC (13 weeks): Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=14; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=13. In bar graphs, data represent number of observations. For the scatter plot, the midline represents the mean ± S.E.M. *P<0.05 by unpaired one-tailed Student's t-test.*

Discussion

We examined whether a pro-inflammatory microbiota accelerates atherogenesis in female *Ldlr$^{-/-}$* mice, a mouse model exhibiting dyslipidemia, inflammation and atherosclerosis when fed a western style diet[20]. We found that *Casp1$^{-/-}$* microbiota increased atherosclerosis in the aortic root in HFC-fed *Ldlr$^{-/-}$* mice (Figure 1A-B). This was accompanied by increased pro-inflammatory plasma cytokines (Figure 3A), increased

blood leukocyte numbers, particularly monocytes and neutrophils (Figure 3B), increased neutrophil accumulation in atherosclerotic plaques (Figure 3C-D), and reduced levels of SCFAs in the cecum (Figure 4E). These results imply a causal relationship between microbiota composition, inflammation, and atherosclerosis.

We found that in particular the plasma levels of IFN-γ, IL-2 and IL-1β were increased in *Ldlr*$^{-/-}$ mice with *Casp1*$^{-/-}$ dysbiosis, suggesting that accelerated atherosclerosis in these mice is partially driven by these cytokines, which is supported by previous studies.[21–23] Furthermore, we showed an increase in peripheral blood leukocytes, which have previously been linked to cardiovascular disease.[24] Within the leukocyte population, neutrophils and monocytes are important contributors to atherogenesis.[25] Increased monocytes and neutrophils in the circulation may lead to infiltration of monocytes and neutrophils into atherosclerotic plaques and further promoting plaque growth.[25]

We observed that exposure to *Casp1*$^{-/-}$ microbiota lowers SCFA-producing taxonomies and cumulative cecum concentrations of SCFAs. SCFAs have anti-inflammatory properties and can suppress NF-kB activity in immune cells,[18] resulting in reduced production of pro-inflammatory cytokines including IFN-γ, IL-1β and IL-2.[26] Furthermore, SCFAs may act as modulators of immune homeostasis by acting as histone deacetylase (HDAC) inhibitors.[19] Oral butyrate supplementation has recently been shown to attenuate the adhesion and migration of macrophages and to decrease pro-inflammatory cytokines in atherosclerotic plaques.[27] Thus, it is tempting to speculate that the reduction in SCFAs in *Ldlr*$^{-/-}$ mice following exposure to *Casp1*$^{-/-}$ microbiota may have contributed to increased levels of pro-inflammatory cytokines and leukocytes in the circulation, and neutrophil accumulation in the atherosclerotic plaque.

It is well recognized that microbial transplantation can be transient.[13] Thus, we cannot exclude that certain effects on TMAO, although not present at time of sacrifice, may have been lost throughout the length of the study. In line with this, the possibility exist that the inflammatory effects may have been dampened over time. Future studies, therefore, should include more frequent and earlier time points to rule out these possibilities.

Whereas previous studies have shown a decreased abundance of *Akkermansia Muciniphila* upon HFD feeding, our data show on opposing

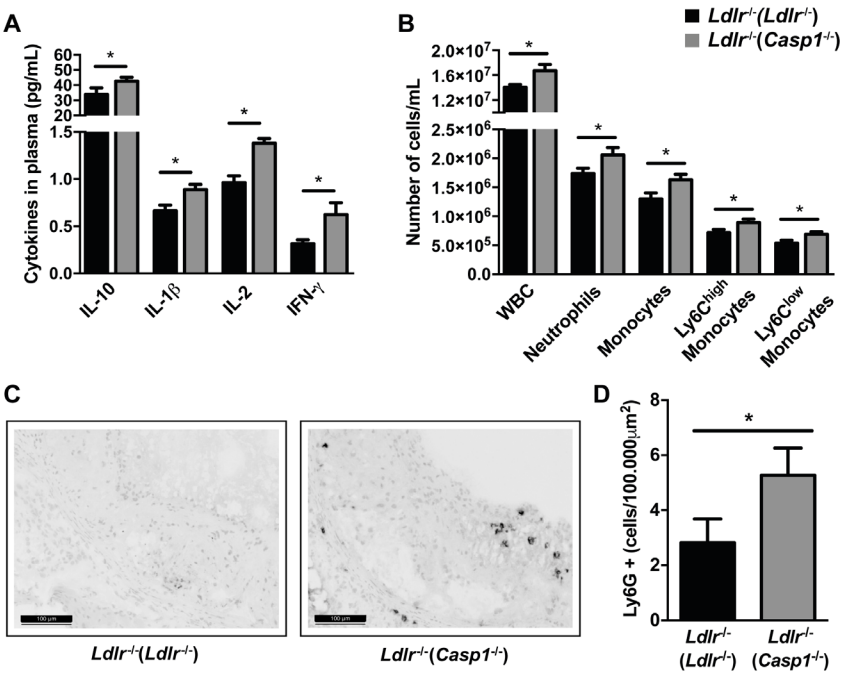


Figure 3 – Casp1$^{-/-}$ dysbiosis leads to systemic inflammation. (**A**). *Plasma cytokines at time of sacrifice. n=10 per group. (B) White blood cell count and immune subsets during week 5 of co-housing. Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=18; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=17.* (**C-D**) *Female Ldlr$^{-/-}$ mice aged 12 weeks were exposed to fecal microbiota derived from Casp1$^{-/-}$ or Ldlr$^{-/-}$ mice for 13 weeks while fed a HFC diet.* (**C**) *Representative Ly6G-stained slides of the aortic root. Scale bars, 100 mm.* (**D**) *Number of infiltrated neutrophils per 100.000 μm^2 characterized by Ly6G-stained slides of the aortic root. Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=13; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=12 Data represent mean ± S.E.M. *P<0.05 as determined by unpaired one-tailed Student's t-test.*

effect on *Akkermansia Muciniphila* following HFC feeding. A similar effect upon high fat and high carbohydrate diet feeding in mice was recently shown [28] and warrants further investigation. Nevertheless, promising results have been obtained with the administration of *Akkermansia Muciniphila* resulting in protection against atherogenesis in *ApoE$^{-/-}$* mice by strengthening the gut barrier and preventing metabolic endotoxemia-induced inflammation.[29] Likewise, metformin's reported beneficial effects on atherosclerosis in humans with type I and II diabetes and non-diabetic

dysglycaemia may be related to its ability to enhance the growth of *Akkermansia Muciniphila* and promote SCFA production.[30] Together with our findings this indicates that manipulation of the gut microbiota composition is an interesting treatment strategy to protect against inflammation and atherosclerosis and reduce cardiovascular disease risk.

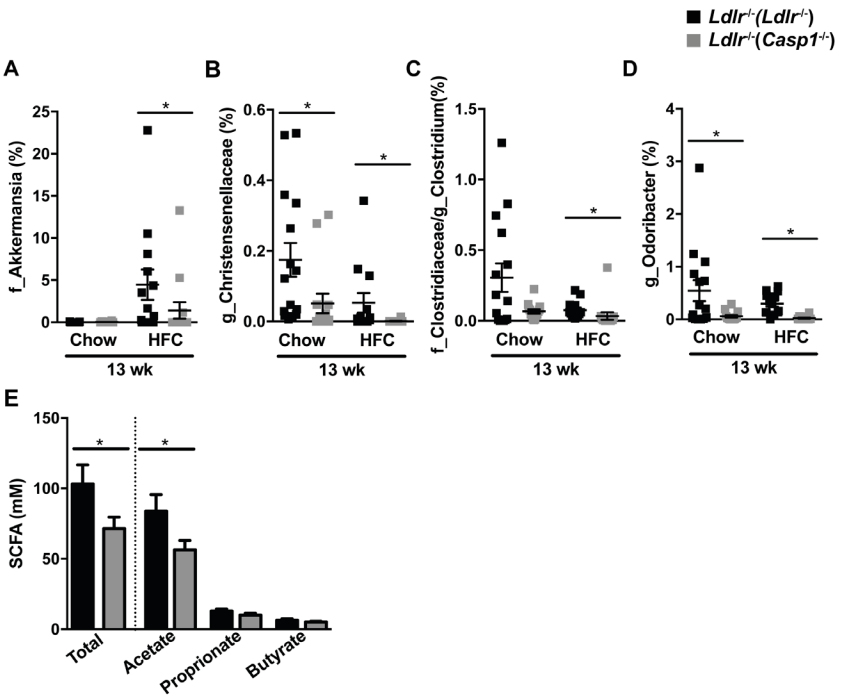


Figure 4 – Casp1$^{-/-}$ induced alterations in the gut microbiota. *Female Ldlr$^{-/-}$ mice were exposed to fecal microbiota derived from Casp1$^{-/-}$ or Ldlr$^{-/-}$ mice by means of co-housing for 13 weeks while fed a chow and HFC diet.* (**A-D**) *Abundance of microbiota taxonomies based on LEfSe analysis of 16S-rDNA-encoding sequences in feces collected at time of sacrifice.* (**A**) *Family Akkermansia.* (**B**) *Genus Christensenellaceae.* (**C**) *Genus Clostridium.* (**D**) *Genus Odoribacter.* (**E**) *Cecum concentration of propionate, acetate, and butyrate in HFC-fed mice.*

(A-D) *Chow: Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=15; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=14; HFC: Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=13; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=14.* **(E)** *Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=8; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=9. Data represent mean ± S.E.M. *P<0.05 as determined by Kruskal-Wallis test* **(A-D)** *and unpaired one-tailed Student's t-test* **(E).**

References

1. Moss JWE, Ramji DP. Cytokines: roles in atherosclerosis disease progression and potential therapeutic targets. Future Med Chem. 2016;8:1317–1330.

2. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–325.

3. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, Fonseca F, Nicolau J, Koenig W, Anker SD, Kastelein JJP, Cornel JH, Pais P, Pella D, Genest J, Cifkova R, Lorenzatti A, Forster T, Kobalava Z, Vida-Simiti L, Flather M, Shimokawa H, Ogawa H, Dellborg M, Rossi PRF, Troquay RPT, Libby P, Glynn RJ. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017;377:1119-1131.

4. Schirmer M, Smeekens SP, Vlamakis H, Jaeger M, Oosting M, Franzosa EA, Jansen T, Jacobs L, Bonder MJ, Kurilshikov A, Fu J, Joosten LAB, Zhernakova A, Huttenhower C, Wijmenga C, Netea MG, Xavier RJ. Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. Cell. 2016;167:1125–1136.e8.

5. Geva-Zatorsky N, Sefik E, Kua L, Pasman L, Tan TG, Ortiz-Lopez A, Yanortsang TB, Yang L, Jupp R, Mathis D, Benoist C, Kasper DL. Mining the Human Gut Microbiota for Immunomodulatory Organisms. Cell. 2017;168:928–943.e11.

6. Khosravi A, Yáñez A, Price JG, Chow A, Merad M, Goodridge HS, Mazmanian SK. Gut microbiota promote hematopoiesis to control bacterial infection. Cell Host Microbe. 2014;15:374–381.

7. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez J-P, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482:179-185.

8. Cani PD, Osto M, Geurts L, Everard A. Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. Gut Microbes. 2012;3:279–288.

9. Karlsson FH, Fåk F, Nookaew I, Tremaroli V, Fagerberg B, Petranovic D, Bäckhed F, Nielsen J. Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat Commun. 2012;3:1245.

10. Jie Z, Xia H, Zhong S-L, Feng Q, Li S, Liang S. The gut microbiome in atherosclerotic cardiovascular disease. Nat Commun. 2017;1–11.

11. Koren O, Spor A, Felin J, Fåk F, Stombaugh J, Tremaroli V, Behre CJ, Knight R, Fagerberg B, Ley RE, Bäckhed F. Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc Natl Acad Sci U S A. 2011;108 Suppl:4592–4598.

12. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, DiDonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WHW, Bushman FD, Lusis AJ, Hazen SL. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013;19:576–585.

13. Gregory JC, Buffa JA, Org E, Wang Z, Levison BS, Zhu W, Wagner MA, Bennett BJ, Li L, DiDonato JA, Lusis AJ, Hazen SL. Transmission of Atherosclerosis Susceptibility with Gut Microbial Transplantation. J Biol Chem. 2015;290:5647–5660.

14. Schiattarella GG, Sannino A, Toscano E, Giugliano G, Gargiulo G, Franzone A, Trimarco B, Esposito G, Perrino C. Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: A systematic review and dose-response meta-analysis. Eur Heart J. 2017.

15. Kasahara K, Tanoue T, Yamashita T, Yodoi K, Matsumoto T, Emoto T, Mizoguchi T, Hayashi T, Kitano N, Sasaki N, Atarashi K, Honda K, Hirata K. Commensal bacteria at the crossroad between cholesterol homeostasis and chronic inflammation in atherosclerosis. J Lipid Res. 2017;58:519–528.

16. Hu B, Elinav E, Huber S, Booth CJ, Strowig T, Jin C, Eisenbarth SC, Flavell RA. Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4. Proc Natl Acad Sci. 2010.

17. Loy A, Pfann C, Steinberger M, Hanson B, Herp S, Brugiroux S, Gomes Neto JC, Boekschoten M V, Schwab C, Urich T, Ramer-Tait AE, Rattei T, Stecher B, Berry D. Lifestyle and Horizontal Gene Transfer-Mediated Evolution of Mucispirillum schaedleri , a Core Member of the Murine Gut Microbiota. mSystems. 2017;2:e00171-16.

18. Tedelind S, Westberg F, Kjerrulf M, Vidal A. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate : A study with relevance to inflammatory bowel disease. 2007;13:2826–2832.

19. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell. 2016.

20. Subramanian S, Han CY, Chiba T, McMillen TS, Wang SA, Haw A, Kirk EA, O'Brien KD, Chait A. Dietary cholesterol worsens adipose tissue macrophage accumulation and atherosclerosis in obese LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol. 2008.

21. Gupta S, Pablo AM, Jiang X c, Wang N, Tall AR, Schindler C. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. J Clin Invest. 1997;99:2752–2761.

22. Upadhya S, Mooteri S, Peckham N, Pai RG. Atherogenic Effect of Interleukin-2 and Antiatherogenic Effect of Interleukin-2 Antibody in Apo-E-Deficient Mice. Angiology. 2004;55:289–294.

23. Swirski FK, Nahrendorf M. Leukocyte Behavior in Atherosclerosis, Myocardial Infarction, and Heart Failure. Science. 2013;339:161–166.

24. Avanzas P, Arroyo-Espliguero R, Cosıan-Sales J, Quiles J, Zouridakis E, Kaski JC. Multiple complex stenoses, high neutrophil count and C-reactive protein levels in patients with chronic stable angina. Atherosclerosis. 2004;175:151–157.

25. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat Rev Immunol. 2008;8:802–815.

26. Klampfer L, Huang J, Sasazuki T, Shirasawa S, Augenlicht L. Inhibition of Interferon; Signaling by the Short Chain Fatty Acid Butyrate. 2003;1:855–862.

27. Aguilar EC, Leonel AJ, Teixeira LG, Silva AR, Silva JF, Pelaez JMN, Capettini LSA, Lemos VS, Santos RAS, Alvarez-Leite JI. Butyrate impairs atherogenesis by reducing plaque inflammation and vulnerability and decreasing NFκB activation. Nutr Metab Cardiovasc Dis. 2014;24:606–613.

28. Carmody RN, Gerber GK, Luevano JM Jr, Gatti DM, Somes L, Svenson KL, Turnbaugh PJ. Diet dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe. 2015; 17, 72–84.

29. Li J, Lin S, Vanhoutte PM, Woo CW, Xu A. Akkermansia muciniphila protects against atherosclerosis by preventing metabolic endotoxemia-induced inflammation in Apoe-/- Mice. Circulation. 2016;133:2434–2446.

30. Rodriguez J, Hiel S, Delzenne NM. Metformin: old friend, new ways of action-implication of the gut microbiome? Curr Opin Clin Nutr Metab Care. 2018.

Supplemental material

The Online Methods

Co-housing strategy and Organ Collection

All animal studies were performed with approval by the University of Groningen Ethical Committee for Animals Experiments, which adheres to the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Experiments were carried out on female *Casp1*$^{-/-}$ mice[1] (a gift from Prof. Netea[2] (B6N.129S2-*Casp1*^{tm1Flv}/J)) and *Ldlr*$^{-/-}$ mice (B6.129S7-*Ldlr*^{tm1Her}/J; Jackson Laboratory, Bar Harbor, ME, US) bred inhouse. Male mice were not used in our co-housing studies to prevent fighting and unnecessary discomfort. Mice were housed in individual ventilated cages and maintained on a 12-hour light/12-hour dark cycle with *ad libitum* access to food and water. At the age of 10-12 weeks, *Ldlr*$^{-/-}$ mice were orally gavaged, once daily, for a period of 10 days with a cocktail of broad spectrum antibiotics (Metronidazole, 0.1 mg/g bodyweight; Ampicillin, 0.26 mg/g bodyweight; Neomycin, 0.26 mg/g bodyweight; Vancomycin, 0.13 mg/g bodyweight) to suppress intestinal microbes. [3-5] Fecal samples were collected before and after the 10-day antibiotic treatment to confirm the effectiveness of the antibiotic treatment. Consistent with previous studies[3,4], total bacterial content was dramatically reduced in *Ldlr*$^{-/-}$ mice that received antibiotics for 10 days (Online Figure IA). At the end of the antibiotic treatment the mice were either kept on chow (RMH-B, AB Diets, the Netherlands) or subjected to a high-fat cholesterol diet (HFC; 60% kcal fat, 0.25% cholesterol, Research Diets, New Brunswick, New Jersey, US). Co-housing did not occur for an additional 7 days as residual antibiotics present in the feces of the antibiotic-treated *Ldlr*$^{-/-}$ mice may, upon consumption, alter the microbiota composition of the donor mice. However, to initiate the colonization process of the gut microbiota towards their donor's composition, we transferred used bedding material (including fecal pellets) from cages housing non-antibiotic-treated *Ldlr*$^{-/-}$ (donor) or *Casp1*$^{-/-}$ (donor) mice to cages housing the antibiotic-treated *Ldlr*$^{-/-}$ mice (recipients) during this first week post-antibiotic treatment (Figure 1A). The antibiotic-treated *Ldlr*$^{-/-}$ mice received new used bedding material from the donor mice, once daily, to have ample supply of fecal pellets for daily consumption. Thereafter, the antibiotic-treated *Ldlr*$^{-/-}$ mice were cohoused with non-

antibiotic-treated *Ldlr*$^{-/-}$ (autologous transplantation) or *Casp1*$^{-/-}$ mice in a 3:2 ratio for a period of 8 or 13 weeks while maintaining the mice on either diets. Successful fecal transplantation was essential for inclusion of mice into the experiment, as determined by 16S sequencing. Mice were excluded in case humane endpoints were reached. Indications of humane endpoints were a decrease of 15% in bodyweight during the experiment, development of scratch marks or signs of discomfort such as a hunchback. All mice met the inclusion criteria and 2 mice from the *Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) HFC 13-wk group and 3 mice from the *Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) HFC 13-wk group were excluded from the experiment because of humane endpoints. Mice were anesthesized by isoflurane during sacrifice and sacrificed by cardiac puncture. Blood was collected in EDTA-coated tubes, spun down at 1000g for 10 min at 4°C, and plasma was stored for further analysis. Aortic arches were removed and frozen in liquid nitrogen. Hearts were embedded in OCT, frozen on dry ice in isopentane and stored at -80°C until further analysis. Intestines were removed and duodenum, ileum, and colon were dissected and snap-frozen in liquid nitrogen or fixated in Carnoy fixative.[6]

16S sequencing

Fresh fecal samples were collected 1 day before sacrifice and immediately snap frozen in liquid nitrogen. Fecal DNA was isolated as previously described[7] and sequenced at the Broad Institute (Boston, MA, US) using Illumina MiSeq paired-end reads. The hypervariable V4 region was amplified using the 515F (GTGCCAGCMGCCGCGGTAA) and 806R primers (GGACTACHVGGGTWTCTAAT). Primer sequences were removed and paired-end reads aligned as previously described.[8] Quality filtering and dereplication of merged pairs was done in Usearch with fastq_maxee set to 1. An Operational Taxonomic Unit (OTU)-table was made by closed reference OTU clustering at 97%, making use of Greengenes13_8 as a reference database. We merged the sequencing data of the following 8 groups into one OTU-table: *Ldlr*$^{-/-}$ (*Ldlr*$^{-/-}$) 13-wk chow diet; *Ldlr*$^{-/-}$ (*Casp1*$^{-/-}$) 13-wk chow diet; *Ldlr*$^{-/-}$ fecal donor mice 13-wk chow diet; *Casp1*$^{-/-}$ fecal donor mice 13-wk chow diet; *Ldlr*$^{-/-}$ (*Ldlr*$^{-/-}$) 13-wk HFC-diet; *Ldlr*$^{-/-}$ (*Casp1*$^{-/-}$) 13-wk HFC-diet; *Ldlr*$^{-/-}$ fecal donor mice 13-wk HFC-diet; *Casp1*$^{-/-}$ fecal donor mice 13-wk HFC-diet. The total number of sequences of this OTU-table was 3474378 with a mean number of 37771

sequences per sample. The negative control to observe the effect of the pro-inflammatory microbiota of the *Casp1*$^{-/-}$ mice on the development of atherosclerosis in *Ldlr*$^{-/-}$ mice were the *Ldlr*$^{-/-}$ mice receiving an autologous fecal transplantation (*Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$). The experiment has been executed during feeding of a chow diet or HFC diet for 13 weeks. Singletons were filtered from the OTU-table and samples were rarefied in the Qiime software package.[9] Unweighted Unifrac analysis (Figure 1B) and bacterial relative abundance (Figure 4A-D, Online Figure VB-D) was determined in Qiime and plotted in R using the function ggplot. Cladograms identifying the most characteristic bacteria for each experimental group were created in LEfSe software.[10]

Flow cytometry

At five weeks after the initial bedding transfer, blood samples were collected by tail bleeding into EDTA-coated tubes and white blood cell (WBC) count was determined by CBC (Medonic CA 620). For analysis of blood leukocytes, tubes were kept at 4°C for the whole procedure unless stated otherwise. Red blood cells were lysed (BD Pharm Lyse, BD Biosciences, San Jose, CA, US) and WBCs were stained with a cocktail of antibodies against CD45-APC-Cy7 (557659, BD Biosciences, San Jose, CA, US), CD115-APC (17-1152-80, eBioscience, San Diego, CA, US) and Ly6C/G-PerCP-Cy5.5 (561103, BD Biosciences, San Jose, CA, US) according to Westerterp et al.[11] Neutrophils were identified as CD45^{+}CD115^{-}Ly6G^{+} and monocytes as CD45^{+}CD115^{+}. Monocytes were further identified as Ly6C^{lo} and Ly6C^{hi} subsets (Figure S1A). Flow cytometry measurements were performed on a BD LSR-II (BD Biosciences, San Jose, CA, US) and analyzed using FlowJo Software.

Analysis of plasma parameters

Plasma triglycerides, total and free cholesterol were determined by commercially available kits (triglycerides and total cholesterol: Roche/ Hitachi, Basel, Switzerland). Cytokines were measured using the Meso Scale Discovery (MSD, Rockville, Maryland, US) V-PLEX pro-inflammatory panel 1 (mouse) kit.

Measurement of TMAO

TMAO, choline, L-carnitine, betaine and γ-butyrobetaine in plasma

were analyzed by ultra-high performance liquid chromatography in combination with isotope dilution tandem mass spectrometry (UPLC-MS/MS). In short, 10 µL plasma was pipetted into 96-well plates, 25 µL internal standard solution was added (containing TMAO-D9, choline-D9, L-carnitine-D3, and betaine-D11), followed by 300 µL 80% acetonitrile (ACN) + 1% formic acid (FA) in Millipore water. The plate was mixed for ten minutes and the content was transferred to an Ostro 96-well plate (Waters, Etten-Leur, the Netherlands). Eluate was collected and diluted with 500 µL 80 % ACN + 1% FA in water and vortexed for one minute. Chromatography with gradient elution was performed with an Acquity UPLC system (Waters, Milford, US) and XBridge BEH Amide column (3.0 mmx. 50 mm, 2.5 µm) at 40 °C. 0.5 µL was injected and flow rate was 0.6 mL/min, with solvent A, 100 mM ammonium formate + 1% FA in water, and solvent B, ACN. The gradient consisted of 10% solvent A and 90% B for 0-0.2 min, followed by a linear gradient to 40% solvent B from 0.2-4.0 min, held at 40% for 0.5 min, and then set at 10% solvent A from 4.5 min to 5.0 min. Total run time was 5 min. Mass spectrometric detection was performed on a XEVO TQ-s system (Waters). Analytes were detected in positive mode and selected reaction monitoring mode. The respective quantifier ion transitions were as follows: m/z 76.15 > 58.3 for TMAO, m/z 104.2 > 60.3 for choline, m/z 162.2 > 103.25 for L-carnitine, m/z 118.2 > 59.3 for betaine, and m/z 146.25 > 60.3 for γ-butyrobetaine. All analytes were baseline separated from each other.

Measurement of short-chain fatty acids

Cecal concentrations of SCFAs were measured as previously described.[12] Briefly, the cecum was thawed and put in a bead beating tube with 100 µl of internal standard (0.5 mg/ml 4-phenyl butyric acid), 20 µl of 20% 5-sulfosalicyclic acid and beads (2.3 mm dia. Zirconia/silica). By bead beating, the entire cecum was disrupted and homogenized for 30 s at 5000 rpm (Precellys 24, Bertin Technologies). After 10 min centrifugation at 18500 g, half of the supernatant was acidified with 10 µl of HCl (37%) and SCFAs were extracted with 2 ml diethylether. After 15 min vortexing and 3 min centrifugation at 1000 g, the supernatant was transferred to a new tube with a tip of spatula of Na_2SO_4, and then vortexed and centrifuged again. Derivatization was performed overnight with 500 µL of the supernatant and 50 µL of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide

(MTBSTFA) at room temperature. Concentrations of the different SCFAs were measured on an Agilent 5975C series GC/MS (Agilent Technologies). The gas chromatograph was equipped with a ZB-1 column (Phenomenex, Torrance, CA, US). Mass spectrometry analysis was performed by electron ionization. Ions monitored were m/z 117 for acetate, m/z 131 for propionate, m/z 145 for butyrate and m/z 221 for 4-phenol butyric acid.

Gut permeability assay and mucus integrity

Gut permeability was measured in unfasted mice at time of sacrifice. Mice received 0.6 mg/g bodyweight of FITC-conjugated dextran (Sigma-Aldrich, St. Louis, US) by oral gavage and blood was collected via cardiac puncture after 4 hr. The concentration of FITC was determined in plasma by fluorometry at 488 nm, dilutions of FITC-dextran in PBS were used as a standard curve. Mucus integrity was determined in cross-sections of colon and ileum (4 μm) and stained with PAS/Alcian blue for detection of the mucus barrier as previously described.[13] Slides were then scanned with a Hamamatsu slide scanner and the thickness of the mucus layer was determined using Image Scope software.

Histological analysis of atherosclerosis

Hearts were cut into sections of 7 μm at the aortic root, after which serial cross-sections of every 42 μm were stained with toluidine blue (0.2% in PBS, Sigma-Aldrich, Gillingham, UK). Slides were scanned with a Hamamatsu slide scanner and plaque size was measured in a blinded fashion using image scope software (Leica Aperio Imagescope, Wetzlar, Germany) and was presented as the sum of 3 valves. Sirius red staining on frozen sections was performed for 30 min to measure collagen content (0.05% direct Red in saturated picric acid, Sigma, Zwijndrecht, the Nederlands). Images were obtained using a Leica DM3000 microscope and quantified using image scope software where collagen was quantified as the percentage of total lesion size. For immunohistochemistry, slides were fixed in acetone and blocked with an Avidin/Biotin Blocking kit (Vector Laboratories). Hereafter, frozen sections were incubated with CD68 antibody (AB53444, Abcam, Cambridge, UK) to stain for macrophages, dectin-1 for alternatively activated macrophages (MCA2289GA, AbD serotec, Uden, The Netherlands), and Ly6G (551459, BD Pharmingen) for neutrophils. Biotin-labeled rabbit anti–rat antibody (CD68: E0468;

a-dectin1, Ly6G: BA-4001, Dako, Eindhoven, the Netherlands) was used as a secondary antibody. CD68-positive, dectin-1-positive and ly6G-positive cells in the lesion area were scored in a blinded fashion and quantified as the percentage of total plaque area or expressed per 100.000 mm^2 (ly6G-positive cells).

Quantitative real-time PCR

Total RNA from aortic arches was isolated using Qiazol reagent and cDNA was synthesized using the Transcriptor Universal cDNA Master kit (Roche, Mannheim, Germany). Real-time PCR was performed with a 7900HT PCR system (Applied Biosystems, Foster city, CA, US) using SYBR Green Master Mix reagent (Roche, Mannheim, Germany). Each sample was run in triplicate and normalized to PPIA as housekeeping gene. We calculated fold changes in gene expression normalized to PPIA by the ΔΔCT method using the equation $2^{-\Delta\Delta CT}$. The results are shown as fold changes compared to the control group. Primer sequences are listed in Supplemental Table I.

Quantification of total bacteria in feces by PCR

Fecal pellets were collected pre-administration (Day 0) and at 10 days after antibiotic administration. Fecal DNA was isolated as previously described[7] and the hypervariable V4 region was amplified using the 515F (GTGCCAGCMGCCGCGGTAA) and 806R primers (GGACTACHVGGGTWTCTAAT).

Statistical analysis

Microbiome composition and abundance

Unweighted Unifrac analysis and bacterial relative abundance was determined in Qiime and plotted in R using the function ggplot. Based on PCA analysis of UniFrac distance matrix, a PERMANOVA analysis using adonis function from R vegan package was then conducted to assess the significance between different mouse groups. Significance was assessed by 1000 permutations. In addition, the LEfse tool developed by the lab of Curtis Huttenhower[10] was used to test for microbial taxonomies that significantly differed in abundance. The algorithm chooses biomarkers (OTU's from the microbiota) by first performing a Kruskall-wallis test

to choose OTU's that are differentially distributed between groups followed by a pairwise Wilcoxon test to test consistency and finally linear discriminant analysis to test the effect size.

Clinical significance after microbiota transfer by means of co-housing
All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5 Software (Graphpad Software, San Diego, CA, US). All data were tested for normality by d'Agastino and Pearson omnibus normality test. We used testing to one-tail since we had postulated an *a priori* hypothesis that an inflammatory microbiota would accelerate atherogenesis. For normally distributed data, Student's T-test was used. For non-normally distributed data, non-parametric Mann-Whitney U test was used. Data were considered significant if $p < 0.05$.

References supplemental material

1. Henao-Mejia J, Elinav E, Jin C, Hao L, Mehal WZ, Strowig T, Thaiss CA, Kau AL, Eisenbarth SC, Jurczak MJ, Camporez J-P, Shulman GI, Gordon JI, Hoffman HM, Flavell RA. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature. 2012;482:179-185.

2. Joosten LAB, Netea MG, Fantuzzi G, Koenders MI, Helsen MMA, Sparrer H, Pham CT, van der Meer JWM, Dinarello CA, van den Berg WB. Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. Arthritis Rheum. 2009;60:3651–3662.

3. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell. 2004;118:229–241.

4. Hill DA, Hoffmann C, Abt MC, Du Y, Kobuley D, Kirn TJ, Bushman FD, Artis D. Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol. 2010;3:148–158.

5. Gregory JC, Buffa JA, Org E, Wang Z, Levison BS, Zhu W, Wagner MA, Bennett BJ, Li L, DiDonato JA, Lusis AJ, Hazen SL. Transmission of Atherosclerosis Susceptibility with Gut Microbial Transplantation. J Biol Chem. 2015;290:5647–5660.

6. Johansson ME V, Hansson GC. Preservation of Mucus in Histological Sections, Immunostaining of Mucins in Fixed Tissue, and Localization of Bacteria with FISH. In: Methods in molecular biology (Clifton, N.J.). 2012. p. 229–235.

7. Schuijt TJ, Lankelma JM, Scicluna BP, de Sousa e Melo F, Roelofs JJTH, de Boer JD, Hoogendijk AJ, de Beer R, de Vos A, Belzer C, de Vos WM, van der Poll T, Wiersinga WJ. The gut microbiota plays a protective role in the host defence against pneumococcal pneumonia. Gut. 2016;65:575–583.

8. Fu J, Bonder MJ, Cenit MC, Tigchelaar EF, Maatman A, Dekens JAM, Brandsma E, Marczynska J, Imhann F, Weersma RK, Franke L, Poon TW, Xavier RJ, Gevers D, Hofker MH, Wijmenga C, Zhernakova A. The Gut Microbiome Contributes to a Substantial Proportion of the Variation in Blood Lipids. Circ Res. 2015;117:817–824.

9. Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. Using QIIME to Analyze 16S rRNA Gene Sequences from Microbial Communities. In: Current Protocols in Bioinformatics. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2011. p. Unit 10.7.-Unit 10.7.

10. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12:R60–R60.

11. Westerterp M, Gautier EL, Ganda A, Molusky MM, Wang W, Fotakis P, Wang N, Randolph GJ, D'Agati VD, Yvan-Charvet L, Tall AR. Cholesterol Accumulation in Dendritic Cells Links the Inflammasome to Acquired Immunity. Cell Metab. 2017;25:1294–1304.e6.

12. den Besten G, Havinga R, Bleeker A, Rao S, Gerding A, van Eunen K, Groen AK, Reijngoud D-J, Bakker BM. The Short-Chain Fatty Acid Uptake Fluxes by Mice on a Guar Gum Supplemented Diet Associate with Amelioration of Major Biomarkers of the Metabolic Syndrome. PLoS One. 2014;9:e107392–e107392.

13. Sovran B, Lu P, Loonen LMP, Hugenholtz F, Belzer C, Stolte EH, Boekschoten M V, van Baarlen P, Smidt H, Kleerebezem M, de Vos P, Renes IB, Wells JM, Dekker J. Identification of Commensal Species Positively Correlated with Early Stress Responses to a Compromised Mucus Barrier. Inflamm Bowel Dis. 2016;22:826–840.

14. Kanters E, Pasparakis M, Gijbels MJJ, Vergouwe MN, Partouns-Hendriks I, Fijneman RJA, Clausen BE, Förster I, Kockx MM, Rajewsky K, Kraal G, Hofker MH, de Winther MPJ. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. J Clin Invest. 2003;112:1176–1185.

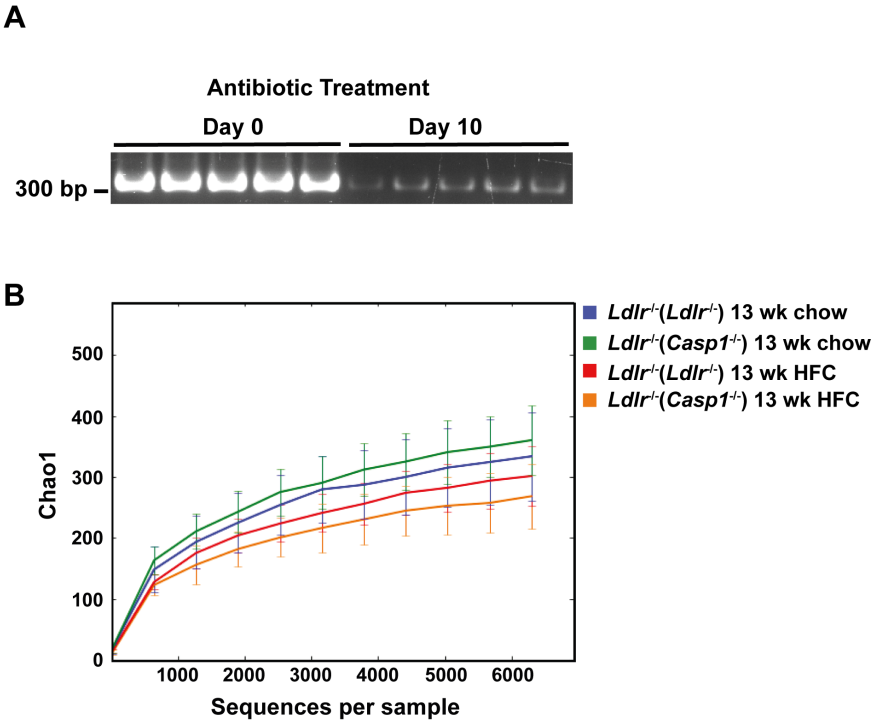
Supplemental figures

Online Table I – Primer sequences

Gene	Forward primer	Reverse primer
Muc2	GGCATCCACTCTAACATCTCCG	CATAGATGGGCCTGTCCTCAGG
Cd68	TGACCTGCTCTCTCTAAGGCTACA	TCACGGTTGCAAGAGAAACATG
Mcp-1	GCTGGAGAGCTACAAGAGGATCA	ACAGACCTCTCTCTTGAGCTTGGT
Tnf-α	GTAGCCCACGTCGTAGCAAAC	AGTTGGTTGTCTTTGAGATCCATG
inos	GCAAAGTCTCAGACATGGCTTG	ATGTCACATGCAGCTTGTCCAG
Icam-1	ACTGCACGTGCTGTATGGTC	CTGCAGGTCATCTTAGGAGATG
Il-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Ccl5	GTGCCCACGTCAAGGAGTAT	CCCACTTCTTCTCTGGGTTG
Mip-2	AGGGCAGGGCAGTAGAATGA	TGTGGCTGGAGTCTGGAGTG
Cxcl-1	CCAAACCGAAGTCATAGCCAC	GTCTTCTTTCTCCGTTACTTGG
Ppia	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG

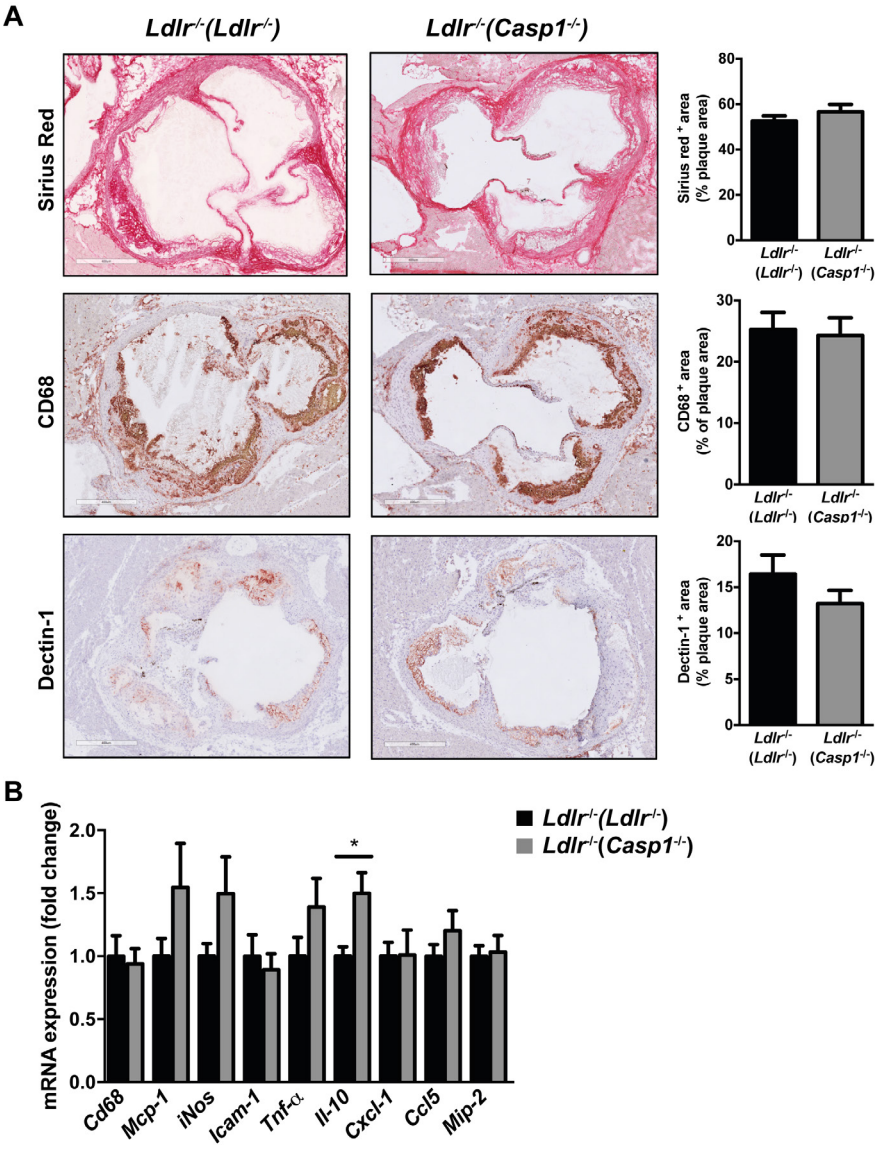
Mouse Groups	*Ldlr*$^{-/-}$ Chow	*Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) Chow	*Ldlr*$^{-/-}$ HFC	*Ldlr*$^{-/-}$(*Ldlr*$^{-/-}$) HFC	*Casp1*$^{-/-}$ Chow	*Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) Chow	*Ldlr*$^{-/-}$(*Casp1*$^{-/-}$) HFC	*Casp1*$^{-/-}$ HFC
Ldlr$^{-/-}$ Chow	NA	0.58	0.001	0.001	0.001	0.001	0.001	0.001
Ldlr$^{-/-}$(*Ldlr*$^{-/-}$) Chow	0.58	NA	0.001	0.001	0.001	0.001	0.001	0.001
Ldlr$^{-/-}$ HFC	0.001	0.001	NA	0.81	0.001	0.001	0.001	0.001
Ldlr$^{-/-}$(*Ldlr*$^{-/-}$) HFC	0.001	0.001	0.81	NA	0.001	0.001	0.001	0.001
Casp1$^{-/-}$ Chow	0.001	0.001	0.001	0.001	NA	0.011	0.001	0.001
Ldlr$^{-/-}$(*Casp1*$^{-/-}$) Chow	0.001	0.001	0.001	0.001	0.011	NA	0.001	0.001
Casp1$^{-/-}$ HFC	0.001	0.001	0.001	0.001	0.001	0.001	NA	0.082
Ldlr$^{-/-}$(*Casp1*$^{-/-}$) HFC	0.001	0.001	0.001	0.001	0.001	0.001	0.082	NA

Online Table II – Related to Figure 1B. p-values for all mouse group comparisons. *Statistical analysis was performed by PERMANOVA analysis based on PCA analysis of UniFrac distance matrix. Significance was assessed by 1000 permutations.*



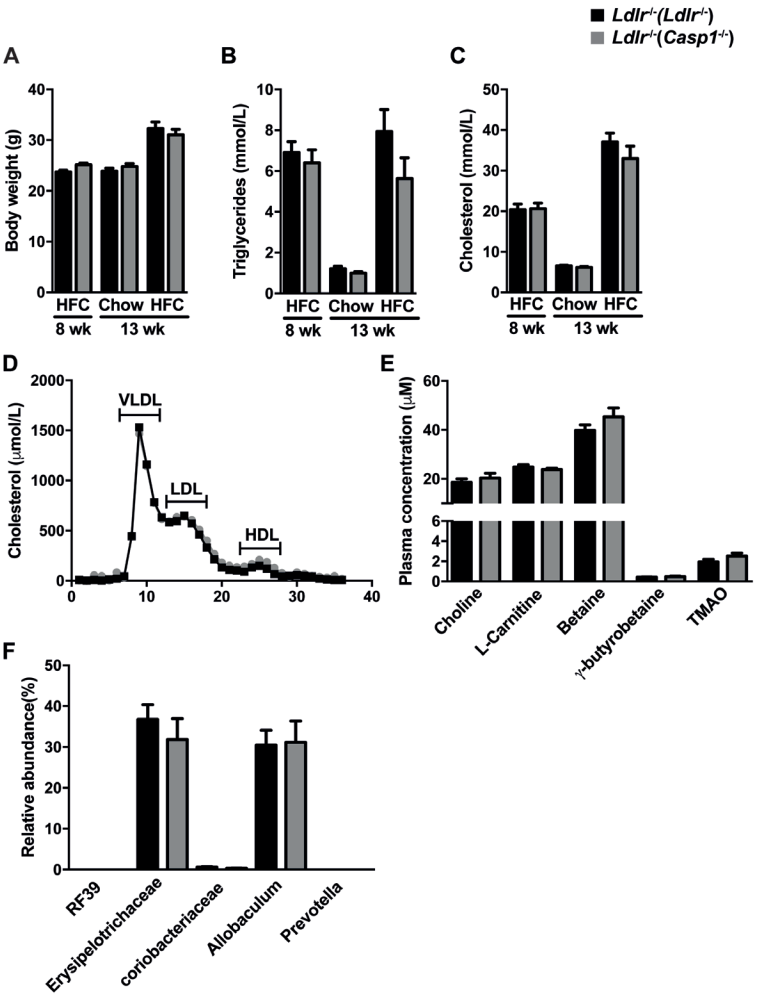
Online Figure I – Total bacterial content following antibiotic treatment in Ldlr$^{-/-}$ mice and a-diversity in co-housed mice

A) *Total bacterial content in feces from Ldlr$^{-/-}$ mice treated with broad spectrum antibiotics (Metrodinazole, Ampicillin, Neomycin, Vancomycin) for 10 days. The PCR bands from 5 representative mice are shown.* **B)** *a-diversity based on 16S-rDNA-encoding sequences in feces collected from chow- and HFC-fed Ldlr$^{-/-}$ mice exposed to Casp1$^{-/-}$ or Ldlr$^{-/-}$ microbiota for 13 weeks.*



Online Figure II – Casp1$^{-/-}$ microbiota does not influence atherosclerotic plaque severity in Ldlr$^{-/-}$ mice fed a HFC diet. *(***A***),* **(left)** *Representative sections of the aortic root stained with Sirius red for collagen content (scale bar, 400 mm), CD68 antibody as macrophage marker (scale bar, 400 mm) and a-dectin-1 antibody for alternatively activated macrophages (scale bar, 400 mm).* **(Right)**

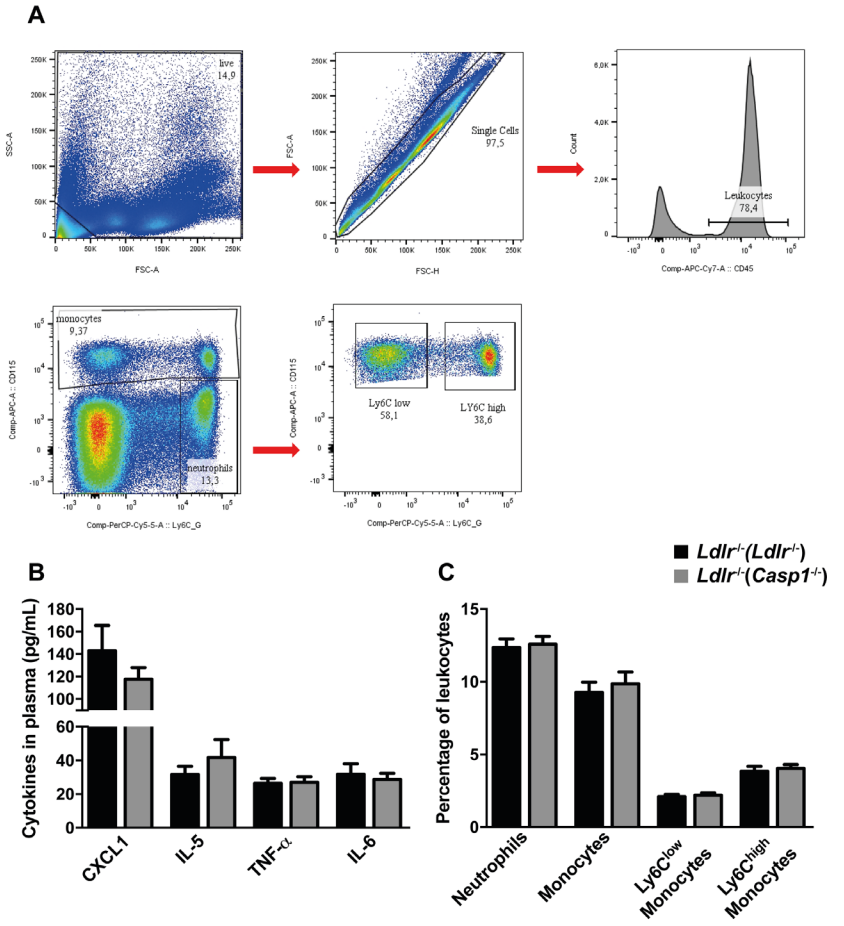
Quantitative analysis of Sirius red-positive area, CD68-positive area and Dectin-1 positive area. (**B**) *Quantitative RT-PCR of mRNAs encoding inflammation and macrophage markers in the aortic arches (n=10 each group).* **HFC (13 weeks)**: *Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=14; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=13. In bar graphs, data represent number of observations. For the scatter plot, the midline represents the mean ± S.E.M. *P<0.05 by unpaired one-tailed Student's t-test.*



Online Figure III – Casp1$^{-/-}$ dysbiosis does not affect plasma lipid and TMAO levels. *Female Ldlr$^{-/-}$ mice aged 12 weeks were exposed to fecal microbiota derived from Casp1$^{-/-}$ or Ldlr$^{-/-}$ mice for 8 or 13 weeks by means of co-housing while fed a chow diet or a HFC diet.* **(A)** *Bodyweight.* **(B)** *Plasma triglycerides.* **(C)** *Plasma*

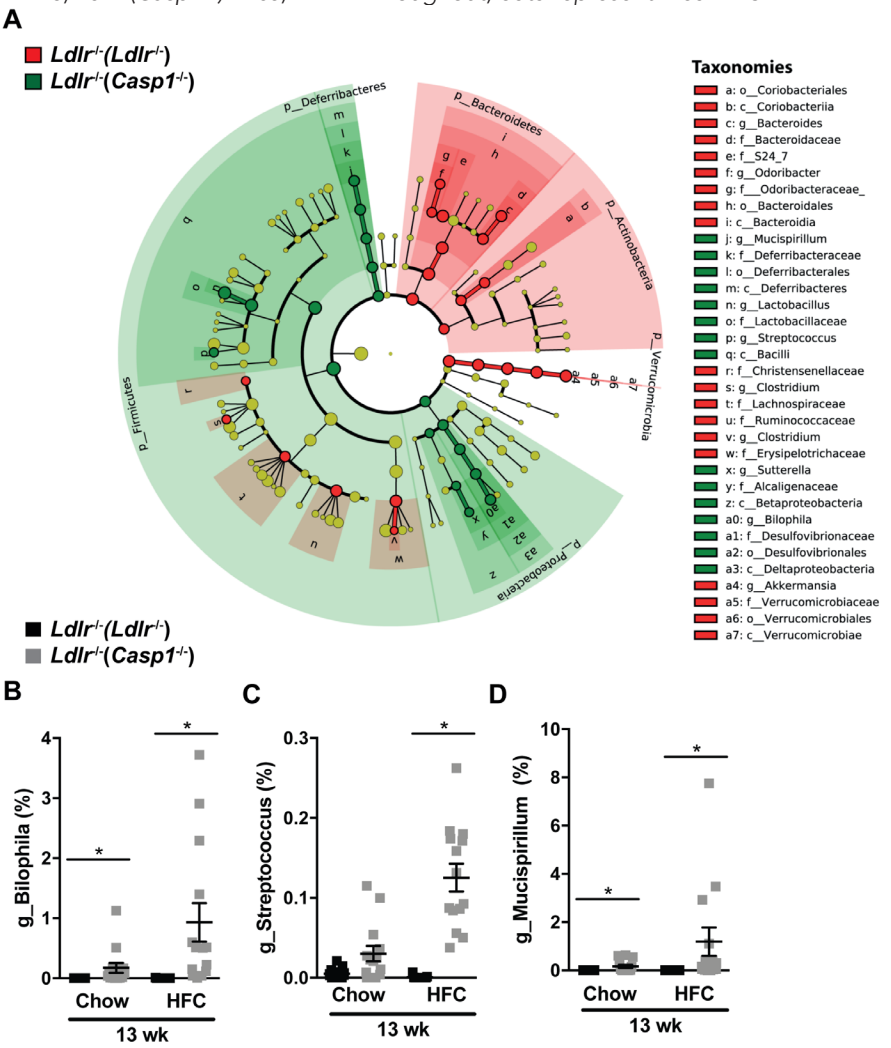
cholesterol. **(D)** *Lipoprotein profile of pooled plasma. (E) Plasma concentration of the TMA precursors choline, L-carnitine, betaine, and g-butyrobetaine and TMAO.* **(F)** *Relative abundance of TMA producing bacteria on the basis of 16S-rDNA-encoding sequences in feces collected at time of sacrifice.*

(A) *Chow (13 weeks): Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=17; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=18; HFC (8 weeks): Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=20; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=19; HFC (13 weeks): Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=16; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=15.* **(B-E)** *n=10 per group.* **(F)** *HFC (13 weeks): (Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=13; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=14. In bar graphs, data represent mean ± S.E.M. ns, not significant as determined by unpaired one-tailed Mann-Whitney U test* **(A-E)**.



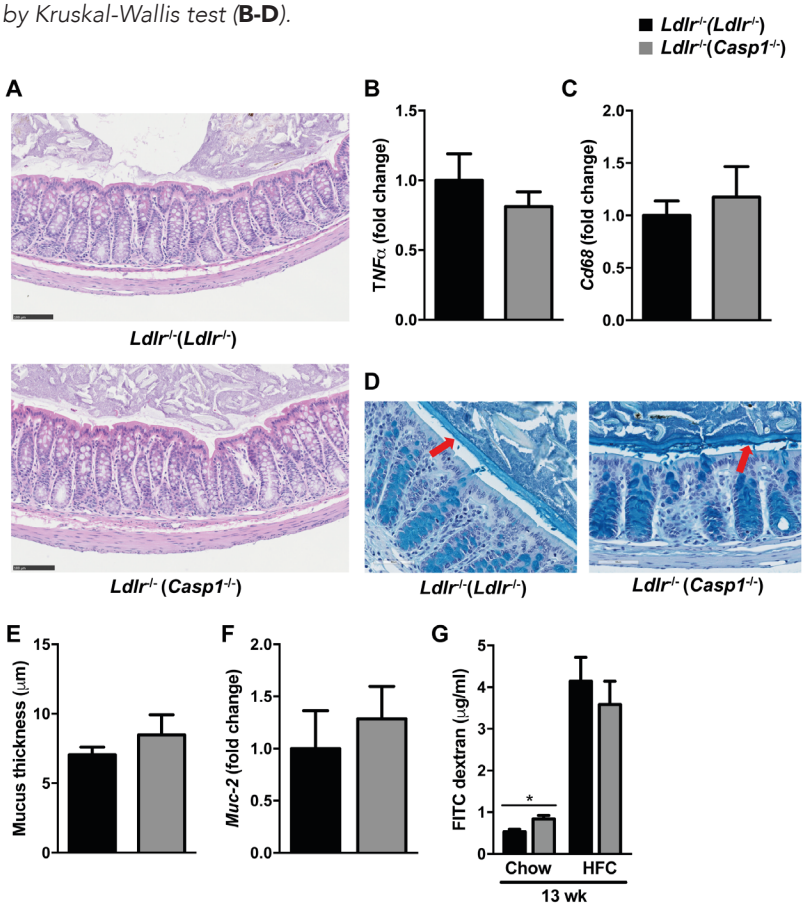
Online Figure IV – Levels of leukocytes and cytokines in blood. (**A-B**) *Female Ldlr$^{-/-}$ mice aged 12 weeks were exposed to a fecal microbiota derived from Casp1^{-}*

*-/- or Ldlr-/- mice by means of co-housing for 13 weeks while fed a HFC diet. (**A**) Gating strategy for the analysis of flow cytometry data. (**B**) Plasma cytokines at time of sacrifice. (**C**) Percentage of blood leukocyte levels during week 5 of the fecal microbiome transplantation. In **B**, n = 10 per group. In **C**, Ldlr-/-(Ldlr-/-) mice, n=18; Ldlr-/-(Casp1-/-) mice, n=19. Throughout, data represent mean ± S.E.M.*



Online Figure V – Casp1-/- induced alterations in the gut microbiota. (**A**) *Female Ldlr-/- mice aged 12 weeks were exposed to fecal microbiota derived from Casp1-/- or Ldlr-/- mice by means of co-housing for 13 weeks while fed a HFC diet. (**A**) Cladogram of LEfSe analysis on the basis of 16S-rDNA-encoding sequences in*

*feces collected at time of sacrifice. Plot shows the 34 microbial taxonomies that differ in abundance following Casp1$^{-/-}$ and Ldlr$^{-/-}$ microbiota transplantation. (***B-D)*** *Abundance of gut microbiota taxonomies based on LEfSe analysis of 16S-rDNA-encoding sequences in feces collected at time of sacrifice. (**B**) Genus Bilophila. (**C**) Genus Streptococcus. (**D**) Genus Mucispirillum.*
*(**A**) Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=13; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=14. (**B-D**) **Chow**: Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=15; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=14; **HFC**: Ldlr$^{-/-}$(Ldlr$^{-/-}$) mice, n=13; Ldlr$^{-/-}$(Casp1$^{-/-}$) mice, n=14. In bar graphs, data represent mean ± S.E.M. *P<0.05 as determined by Kruskal-Wallis test (**B-D**).*



Online Figure VI – Casp1$^{-/-}$ dysbiosis does not result in impaired gut integrity. *(**A-G**) Female Ldlr$^{-/-}$ mice aged 12 weeks were exposed to fecal microbiota derived from Casp1$^{-/-}$ or Ldlr$^{-/-}$ mice by means of co-housing for 13 weeks while fed a HFC*

*diet. (**A**) Representative colon histology by H&E staining at 20x magnification (scale bar, 100 mm). (**B**) Tnfα and (**C**) Cd68 expression in colon. (**D**) Representative PAS/Alcian blue stained mucus layer of the colon at 40x magnification (scale bar, 60 mm). (**E**) Quantification of the mucus layer thickness of the colon. (**F**) Muc-2 expression in colon. (**G**) FITC-labeled dextran in plasma. In **A-C, F-G**, n = 10 per group. In **D-E**, n=8 per group. Throughout, data represent mean ± S.E.M.*

CHAPTER 4

A protective role for the antimicrobial peptide REG3γ in atherogenesis

Eelke Brandsma[1], *Niels Kloosterhuis[1], Mirjam Koster[1], Daphne Dekker[1], Arnau Vich[2], Niels Mulder[1], Marten Hofker[1]†, Bart van de Sluis[1]* and Debby Koonen[1]**

† Deceased
**Equal contribution*

1.University of Groningen, University Medical Center Groningen, Department of Pediatrics, Groningen, the Netherlands.
2.University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, the Netherlands.

Abstract

Background

A role for the gut microbiota in the development of cardiovascular disease has recently been established. Therefore, understanding the mechanisms controlling the gut microbiota could be essential to identify factors in protection against atherogenesis. The production of mucus and excretion of antimicrobial peptides in the gut forms the first line of defense against infiltration of the gut microbiota into the intestinal epithelial barrier. Thus, antimicrobial peptides could play a protective role in atherogenesis by preventing infiltration of the gut microbiota into the intestinal epithelial barrier. Here, we investigated the contribution of the antimicrobial peptide REG3γ in the susceptibility to atherogenesis in mice.

Methods

We overexpressed a PCSK9 gain-of-function mutant in the liver of female Reg3$\gamma^{-/-}$ and WT littermate mice via an AAV-delivery system and fed these mice a high-fat, high-cholesterol diet (HFC) for 11 weeks. During time of sacrifice heart, aortic arch, blood, intestine and fecal samples were collected to determine atherosclerosis development.

Results

Hepatic expression of PCSK9 gain-of-function mutant resulted in a strong reduction in the LDL receptor (LDLR) in the liver, which coincided with hypercholesterolemia in both *Reg3*$\gamma^{-/-}$ and WT mice. Analysis of atherosclerotic lesion size in the aortic root demonstrated a 28% increase in lesion size in *Reg3*$\gamma^{-/-}$ mice with respect to WT littermates. These effects are independent of alterations in blood lipid levels, intestinal permeability or alterations in microbiome composition.

Conclusions

The antimicrobial peptide REG3γ plays a protective role against the development of atherosclerosis.

Introduction

Atherosclerosis is one of the leading causes of death worldwide with a multifactorial etiology that includes a role for genetics, dietary intake and inflammation (Krauss et al., 2000; Libby et al., 2011; Lusis et al., 2004). Recently, the gut microbiota has been identified as an additional player in atherogenesis (Koeth et al., 2013; Li et al., 2016; Tang and Hazen, 2014; Chapter 3). Both a disturbance in microbiota composition (Koren et al., 2011; Ott et al., 2006) and in the production of microbiota-derived metabolites, such as trimethylamine-oxide (TMAO) and short-chain fatty acids (SCFA's) (Aguilar et al., 2014; Koeth et al., 2013) has been implicated in atherogenesis.

Studies have shown that increased infiltration of luminal bacteria into the intestinal epithelial barrier results in the translocation of bacteria or endotoxins across the intestinal epithelial lining into the systemic circulation (Wang et al., 2016). This increase in endotoxins and bacteria into the systemic circulation is linked to the atherosclerotic disease process (Koren et al., 2011; Li et al., 2016). In addition, taxonomies of the gut microbiota have been found in atherosclerotic plaques of patients with symptomatic atherosclerosis (Koren et al., 2011; Ott et al., 2006) and injection of LPS has been shown to exacerbate atherogenesis in *ApoE*$^{-/-}$ mice (Yin et al., 2013). Taken together, these findings indicate that controlling the infiltration of luminal bacteria into the intestinal epithelial barrier could be of importance in protecting against atherosclerosis.

The epithelial lining of the intestine provides the first line of defense against the infiltration of bacteria from the gut lumen into the intestinal epithelial barrier (Brandsma et al., 2015; Loonen et al., 2013; Vaishnava et al., 2011; Wang et al., 2016). This physical-chemical barrier consists of a mucus layer in which Paneth cells excrete antimicrobial peptides, such as the C-type lectin REG3γ. REG3γ prevents the infiltration of gram-positive bacteria into the intestinal epithelial barrier and Reg3γ$^{-/-}$ mice display enhanced infiltration of gram-positive bacteria into the intestinal epithelial barrier (Loonen et al., 2013; Vaishnava et al., 2011; Wang et al., 2016). Several studies have reported the importance of these antimicrobial peptides, including REG3γ in the protection against leakage of endotoxins or bacteria from the gut lumen into the systemic circulation in the development of the metabolic syndrome (Su et al., 2016; Veilleux et al., 2015; Wang et al., 2016). However, it has not been studied whether

increased infiltration of the gut microbiota into the intestinal epithelial barrier plays a role in atherogenesis. Here, we used *Reg3γ*$^{-/-}$ mice to investigate the importance of bacterial infiltration into the intestinal epithelial barrier to the atherosclerotic disease process. We show that atherogenesis is accelerated in *Reg3γ*$^{-/-}$ mice, indicating that preventing bacterial infiltration is an important factor in controlling atherogenesis.

Material and Methods

AAV-production

HEK293T cells were transfected with helper plasmid, adenoviral plasmid and pAAV/D377Y-mPCSK9 plasmid in a 2:1:1 ratio using polyethylenimine transfection. Cells were harvested after 60 hours of culturing at 37C, 5% CO2. The recombinant PCSK9-gain-of-function-AAV (PCSK9-GOF-AAV) was isolated from the cells by two freeze-thaw cycles. PCSK9-GOF-AAV was purified from the lysate by iodixanol centrifugation, desalted, concentrated by spin filter and stored at -80C.

Animal experiment

All animal studies were performed with approval by the University of Groningen Ethical Committee for Animals Experiments, which adheres to the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Experiments were carried out on female *Reg3γ*$^{-/-}$ mice (B6.129-Reg3gtm1.1Lvh/J; Jackson Laboratory, Bar Harbor, USA, ME) and WT littermates, bred inhouse. Mice were housed in groups in Individual Ventilated Cages and maintained on a 12-hour light/12-hour dark cycle with ad libitum access to food and water. *Reg3γ*$^{-/-}$ mice and WT littermates were injected via the orbital vein with 2.0x1010 vector genomes of PCSK9-GOF-AAV at 12 weeks of age (Bjorklund et al., 2014). The diet was switched from standard chow to high-fat cholesterol diet (HFC; 60% kcal fat, 0.25% cholesterol, Research Diets) 2 days after AAV-injection. *Reg3γ*$^{-/-}$(PCSK9) and WT(PCSK9) mice were fed a HFC diet for 11 weeks. Blood was collected after 2 and 7 weeks to measure plasma lipid levels. Fecal samples were collected on the day prior to sacrifice. Mice were sacrificed using cardiac puncture, blood was collected in EDTA-coated tubes, spun down at 1000g for 10 min at 4ºC and plasma was stored for further analysis. Aortic arches were removed and frozen in liquid

nitrogen. Hearts were embedded in OCT, frozen on dry ice in isopentane and stored at -80°C until further analysis. Intestines were removed and duodenum, ileum, and colon were dissected and snap-frozen in liquid nitrogen or fixated in carnoy fixative (Johansson and Hansson, 2012).

Fecal transplantation experiment

The microbiota of *Casp1*$^{-/-}$ mice (a gift from Prof. Netea (Joosten et al., 2009) (B6N.129S2-Casp1tm1Flv/J)) was transplanted into *Reg3γ*$^{-/-}$ and WT mice (Fig 4B). Fecal transplantation was performed as previously described (Chapter 3). In short, at the age of 10 weeks mice were treated with antibiotics for 10 days, followed by transfer of bedding from *Casp1*$^{-/-}$ mice into the cages of *Reg3γ*$^{-/-}$ or WT mice for 7 days. Mice were then injected with 2.0x1010 vector genomes of PCSK9-GOF-AAV (Bjorklund et al., 2014). The diet was switched from a standard chow diet to a HFC diet 2 days after AAV-injection and *Reg3γ*$^{-/-}$(PCSK9) and WT(PCSK9) mice remained on this diet for 13 weeks. Blood was collected at week 2 and week 7 after the start of the HFC intervention to determine plasma lipid levels and mice were sacrificed at week 13 of HFC feeding and organs were collected as described above.

Microbiota composition

Microbiota composition was determined as described in Chapter 3. In short, fecal samples were collected 1 day prior to sacrifice and immediately snap-frozen, fecal DNA was isolated and sequenced using Illumina MiSeq paired-end reads. Amplicons were generated by 16S sequencing targeting the hypervariable V4 region. An OTU-table was made by closed-reference OTU-picking at 97% making use of Greengenes13_8 reference database. Weighted Unifrac distances were determined in Qiime and plotted into PCoa plots in R. Determination of statistically significantly different taxonomies was classified by MaAsLin analysis (Morgan et al., 2012).

Analysis of plasma parameters

Plasma triglycerides and total cholesterol were determined by commercially available kits (Roche/Hitachi, Basel, Switzerland).

Gut permeability Assay

Gut permeability was measured in fed mice at time of sacrifice. Mice received 0.6 mg/g bodyweight of FITC-conjugated dextran (Sigma-Aldrich, St Louis, USA) by oral gavage and blood was collected via cardiac puncture after 4 hr. The concentration of FITC was determined in plasma by fluorometry at 488 nm as described in Chapter 3.

Histological analysis of atherosclerosis

Hearts were cut into sections of 7 μm at the aortic root, after which serial cross-sections of every 42 μm were stained with toluidin blue. Slides were scanned with a Hamamatsu slide scanner and plaque size was measured in a blinded fashion using image scope software (Leica Aperio Imagescope, Wetzlar, Germany) and was presented as the sum of 3 valves.

Quantitative real-time PCR

Total RNA from aortic arches was isolated using Qiazol reagent and cDNA was synthesized using the Transcriptor Universal cDNA Master kit (Roche, Mannheim, Germany). Real-time PCR was performed with a 7900HT PCR system (Applied Biosystems, Foster city, CA, US) using SYBR Green Master Mix reagent (Roche, Mannheim, Germany). Each sample was run in triplicate and normalized to PPIA as housekeeping gene. We calculated fold changes in gene expression normalized to PPIA by the ΔΔCT method using the equation 2-ΔΔCT. The results are shown as fold changes with respect to the compared to the control group. Primer sequences are listed in Supplemental Table 1.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5 Software (Graphpad Software, San Diega, CA, USA). All data were tested for normality by d'Agastino and Pearson omnibus normality test. For normally distributed data Student's T-test was used. For non-normally distributed data, non-parametric Mann-Whitney U test was used. Data were considered significant if $p < 0.05$.

Results

PCSK9-GOF-AAV injection promotes hyperlipidemia

To understand whether infiltration of bacteria into the intestinal epithelial barrier is involved in atherogenesis, we first injected *Reg3γ*$^{-/-}$ and WT littermate mice with 2.0x1010 particles of adeno-associated virus (AAV) containing a PCSK9 gain-of-function mutant (AAV-PCSK9-GOF). *Reg3γ*$^{-/-}$(PCSK9) and WT(PCSK9) mice were then fed a HFC-diet for 11 weeks to induce atherogenesis (Bjorklund et al., 2014; Ishibashi et al., 1993). Injection with AAV-PCSK9-GOF resulted in massive reduction of LDLR in the livers of *Reg3γ*$^{-/-}$ and WT mice (Figure 1A, B), reaching levels which are comparable to LDLR levels in *Ldlr* knockout (*Ldlr*$^{-/-}$) mice (Figure 1A, B). As a result, hepatic expression of PCSK9-GOF raised plasma cholesterol levels over time (Figure 1C) and to levels equivalent to *Ldlr*$^{-/-}$ mice fed a HFC-diet (Figure 1D). Thus, AAV-PCSK9-GOF injection together with HFC feeding created a hyperlipidemic mouse model comparable to the atherosclerosis-prone *Ldlr*$^{-/-}$ mice. We observed no differences in plasma total cholesterol levels (Figure 1C) and in the distribution of cholesterol among lipoprotein particles (Figure 1E) between *Reg3γ*$^{-/-}$(PCSK9) and WT(PCSK9) mice. Furthermore, ablation of Reg3γ did not affect body weight (Figure 1F) and plasma triglyceride levels (Figure 1G), indicating that metabolic parameters are similar between the two groups.

Increased bacterial infiltration promotes atherosclerosis

Next, we assessed atherosclerosis development in both groups by analyzing the atherosclerotic lesion size in the aortic root of HFC-diet fed *Reg3γ*$^{-/-}$(PCSK9) and WT(PCSK9) mice. Atherosclerotic lesion size was increased by 28% in *Reg3γ*$^{-/-}$(PCSK9) mice compared to WT(PCSK9) mice (Figure 2A, B; $p<0.05$). This was not accompanied by increased expression of the inflammatory genes *Tnfα*, *Mcp-1*, *Icam-1*, *iNos* and *Cd68* in the aortic arch of *Reg3γ*$^{-/-}$(PCSK9) with respect to WT(PCSK9) mice (Figure 2C-G). Thus, *Reg3γ*$^{-/-}$(PCSK9) mice show an increase in atherosclerosis development in the aortic root, without affecting inflammatory gene expression in the aortic arch.

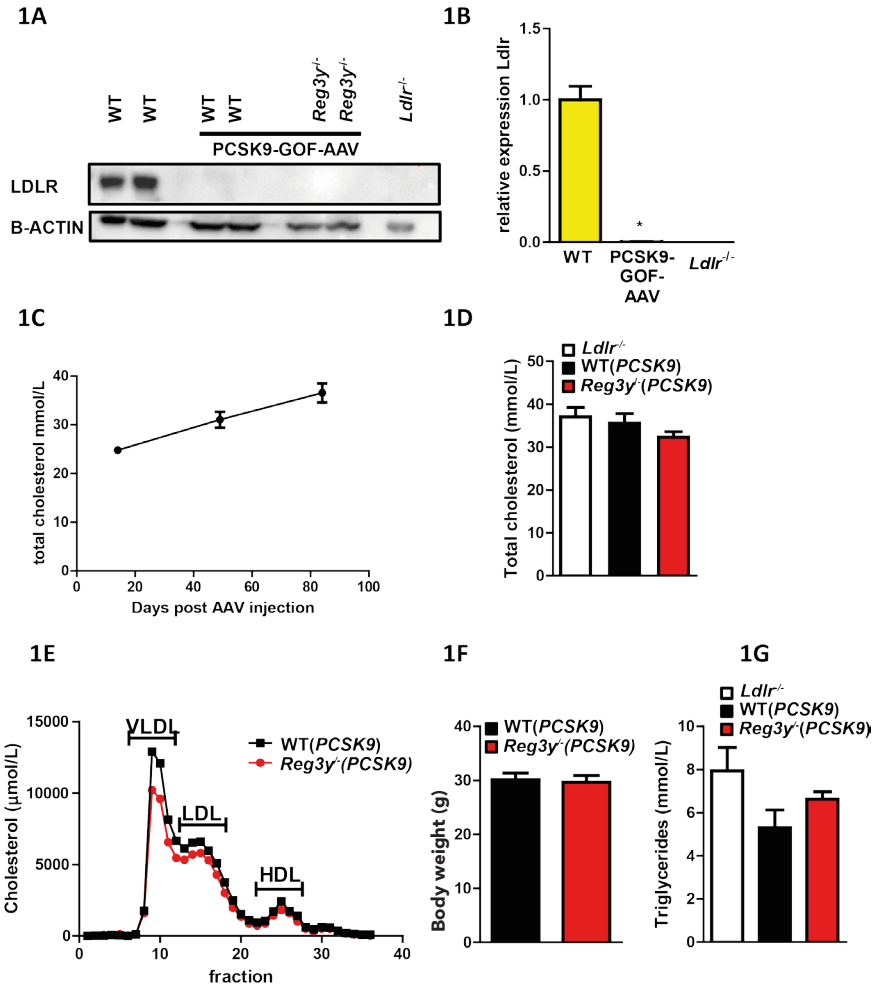


Figure 1 - Blood lipid levels are not altered in Reg3γ-/- mice.

*Female Reg3γ-/- mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) Gene expression analysis of Reg3γ. (B) Western blot analysis of LDLR in liver. (C) Quantification of western blot analysis. (D) Plasma total cholesterol concentrations over time. (E) Total cholesterol in plasma at t=11 weeks. (F) Body weight, (G) Triglycerides in plasma at t=11 weeks (H) FPLC profile at t=11 weeks. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired two-tailed Student's t-test.*

Increased atherogenesis in *Reg3γ*$^{-/-}$ mice is independent of intestinal permeability

To understand whether the enhanced atherogenesis in *Reg3γ*$^{-/-}$(PCSK9) mice is caused by aberrant intestinal health, we analyzed intestinal inflammation and permeability. Histological analysis of the ileum showed no inflammatory phenotype in *Reg3γ*$^{-/-}$(PCSK9) compared to WT(PCSK9) mice (Figure 3A, B). This was further confirmed by gene expression analysis of inflammatory markers in the ileum, which showed that Reg3γ deficiency does not affect the expression of the pro-inflammatory genes *Mcp1*, *Ifn-γ*, *IL1β*, *Tnf-α* and *Ccl5* (Figure 3C-H). In addition, intestinal permeability, as measured by the FITC-dextran (FD4) in vivo permeability assay, was not affected by depletion of Reg3γ (Figure 3F). These results indicate that intestinal barrier function is not compromised in *Reg3γ*$^{-/-}$ (PCSK9) mice and thus cannot explain the increase in atherogenesis seen in these mice.

***Reg3γ*$^{-/-}$ mice do not display microbiota dysbiosis**

To exclude a role for a disturbed gut microbiota composition as driving factor for the increased susceptibility to atherogenesis in *Reg3γ*$^{-/-}$(PCSK9) mice, we analyzed fecal microbiota composition of HFC-diet fed *Reg3γ*$^{-/-}$ (PCSK9) and WT(PCSK9) mice by 16S rDNA sequencing. Weighted UniFrac distances of 16S rDNA sequences demonstrated clustering between the *Reg3γ*$^{-/-}$(PCSK9) mice, whereas the microbiome of WT(PCSK9) mice was more diverse (Figure 4A). To understand whether there are specific taxonomies that are altered between *Reg3γ*$^{-/-}$(PCSK9) mice with respect to WT(PCSK9) mice, we investigated the microbiome on the genus level, and found no major differences in the top-20 of most abundant taxonomies (Figure 4B). In addition, Maaslin analysis failed to detect statistical significant differences between the taxonomies of the gut microbiome and the genotype of the mice indicating that the microbiota composition is not markedly altered by the loss of REG3γ (Figure 4C).

AAV-PCSK9-GOF injection does not result in hepatic LDLR knockdown and a hyperlipidemic mouse model

As our data emphasizes a protective role for REG3γ in atherogenesis, we next assessed whether a microbiome harboring bacterial species prone

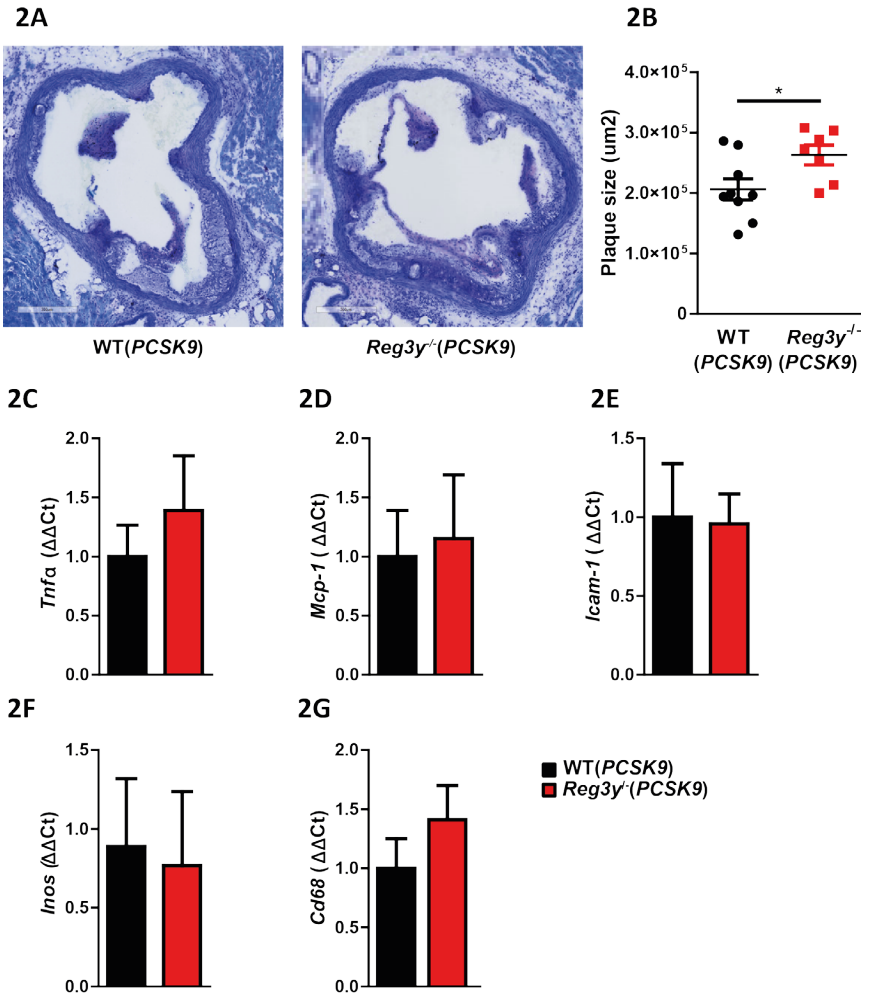


Figure 2 - Increased bacterial infiltration promotes atherosclerosis

*Female Reg3γ-/- mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) Representative images of the aortic root stained by toluidin blue for determination of the lesion area. (B) Quantification of atherosclerotic root lesion area. (C-G) Gene expression in the aortic arch. (C) Tnfα. (D) Mcp-1. (E) Icam-1. (F) Inos. (G) Cd68. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired one-tailed Student's t-test.*

to infiltrate the intestinal epithelial barrier (e.g. mucispirillum schaedleri, Attaching Invading Eschericia Coli) (Loy et al., 2017; Martinez-Medina et al., 2014) is able to further advance atherogenesis in *Reg3γ*$^{-/-}$(PCSK9) mice. We have recently shown that *Ldlr*$^{-/-}$ mice transplanted with the pro-inflammatory microbiome of *Caspase 1*$^{-/-}$ (*Casp1*$^{-/-}$) mice display an increased abundance of the taxonomies *Bilophila*, *Streptococcus* and *Mucispirillum* (Chapter 3). As these mice show advanced atherogenesis (Chapter 3), we hypothesized that atherogenesis would be more severe

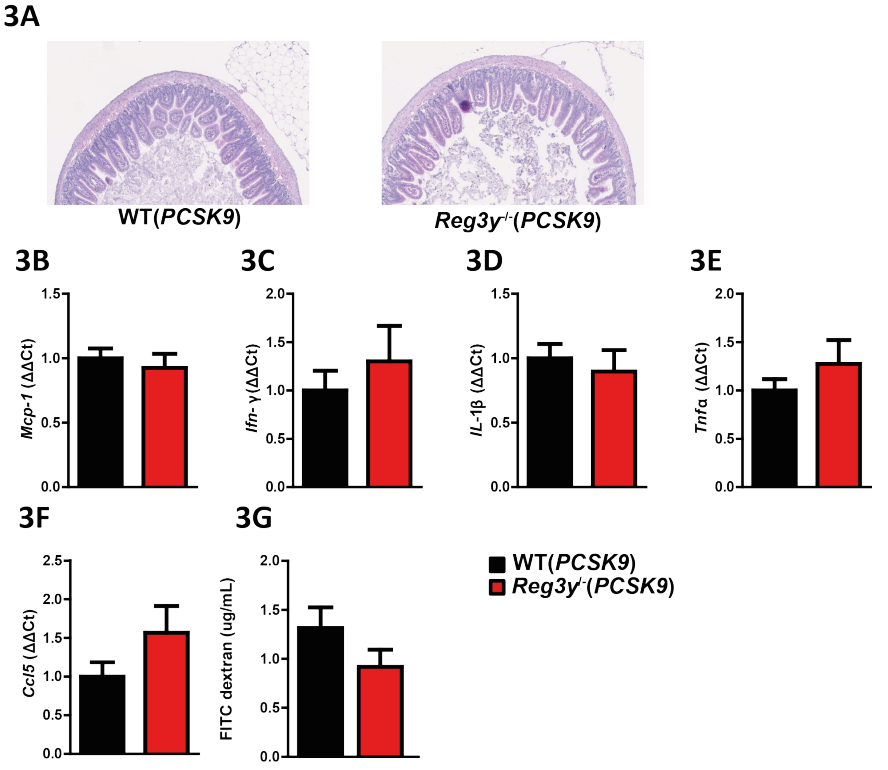


Figure 3 – Intestinal inflammation and permeability is not affected in Reg3γ-/- mice.

*Female Reg3γ-/- mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) H&E staining of ileum. (B-F) Gene expression analysis of inflammatory markers in the ileum. (B) Mcp-1. (C) Ifn-γ. (D) Il-1β. (E) Tnf-α. (F) Ccl5. (G) FITC dextran in vivo permeability assay. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired one-tailed Student's t-test.*

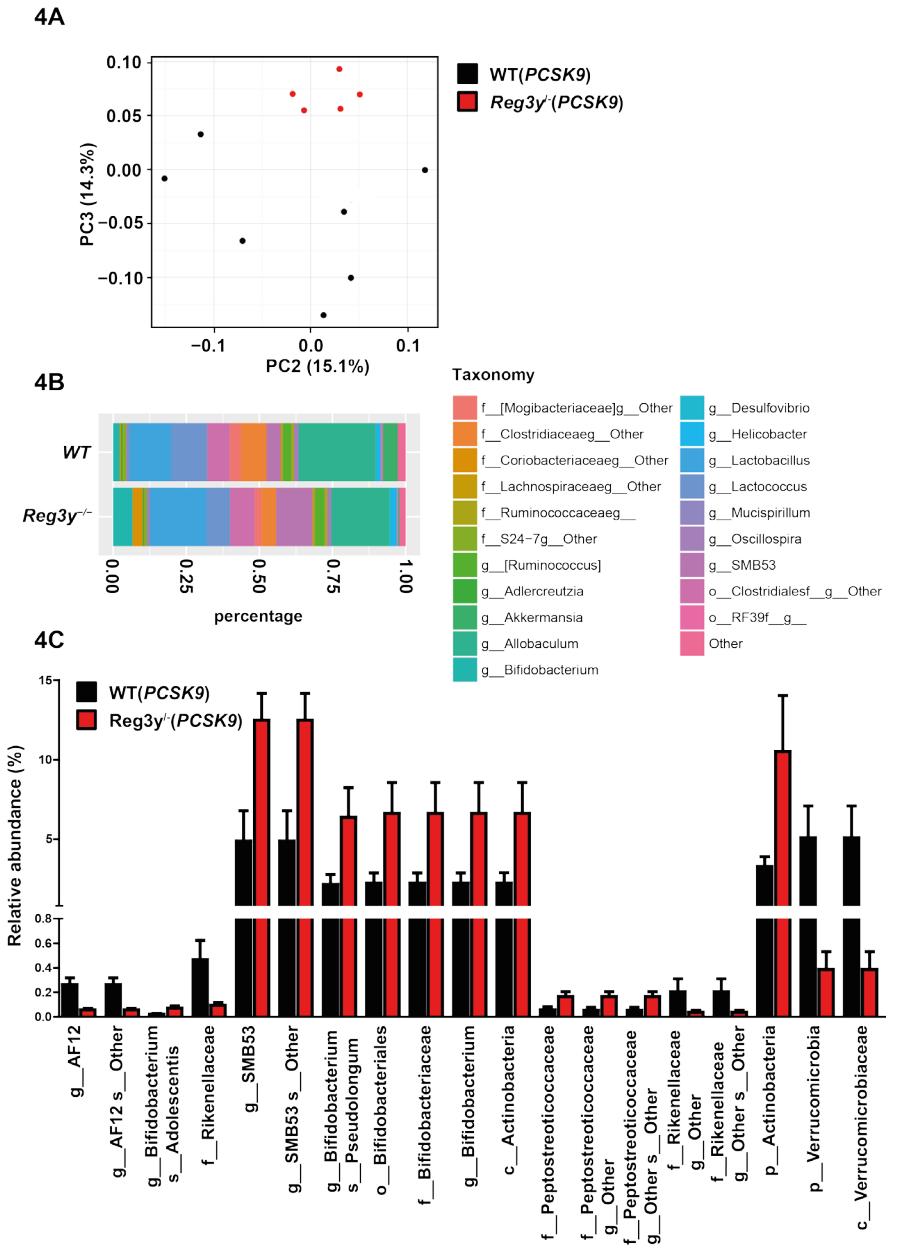


Figure 4 – Reg3γ-/- mice do not show disturbances in microbiota composition.

Female Reg3γ-/- mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) Principal-coordinate analysis plot of Weighted

in *Casp1*$^{-/-}$ microbiome-transplanted *Reg3γ*$^{-/-}$(PCSK9) mice compared to *Casp1*$^{-/-}$ microbiome-transplanted WT(PCSK9) mice. Thus, we performed a fecal microbiota transplantation in *Reg3γ* and WT littermate mice (Figure 5A) and transduced the mice with a PCKS9-GOF-AAV to provoke atherogenesis under HFC-feeding conditions. A new batch of AAV-PCSK9-GOF was generated, however, this virus batch did not result in depletion of hepatic LDLR (Figure 5B, C) and hypercholesterolemia (Figure 5D-F) in *Casp1*$^{-/-}$ microbiome-transplanted *Reg3γ*$^{-/-}$(PSCK9) and WT (PCSK9) mice. As a consequence, atherosclerosis development could not be studied in *Casp1*$^{-/-}$ microbiome-transplanted *Reg3γ*$^{-/-}$(PSCK9) and WT (PCSK9) mice, since it is very unlikely that atherosclerotic plaques have developed under these conditions (Ishibashi et al., 1993).

Discussion

To explore whether infiltration of luminal bacteria into the intestinal epithelial barrier plays an important role in the atherosclerotic disease process, we investigated atherogenesis in *Reg3γ*$^{-/-}$ and WT littermate mice expressing a PCSK9-GOF mutant and fed a HFC-diet for 11 weeks to provoke atherogenesis. Our data show that atherogenesis is increased by 28% in *Reg3γ*$^{-/-}$(PCSK9) compared to WT(PCSK9) mice (Figure 2A-B; $p<0.05$). This was not associated with a difference in plasma lipid levels (Figure 1E,G), impaired intestinal permeability (Figure 3G) and gut microbiota dysbiosis (Figure 4C). Our findings unravel an athero-protective role for REG3γ and together with previous reports identifying a protective role for antimicrobial peptides in the etiology of the metabolic syndrome (Su et al., 2016; Veilleux et al., 2015; Wang et al., 2016) emphasize the importance of antimicrobial peptides in the prevention of cardio-metabolic diseases.

It has previously been shown that infiltration of bacteria into the intestinal epithelial barrier can contribute to increased intestinal inflammation

*UniFrac distance on the basis of 16S-rDNA-encoding sequences in feces collected from Reg3γ-/- mice and WT littermates fed a HFC diet for 11 weeks. (B) Stacked bar graph representing the 20 most abundant taxonomies. (C) Top 20 most different taxonomies from MAaslin analysis. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired one-tailed Student's t-test.*

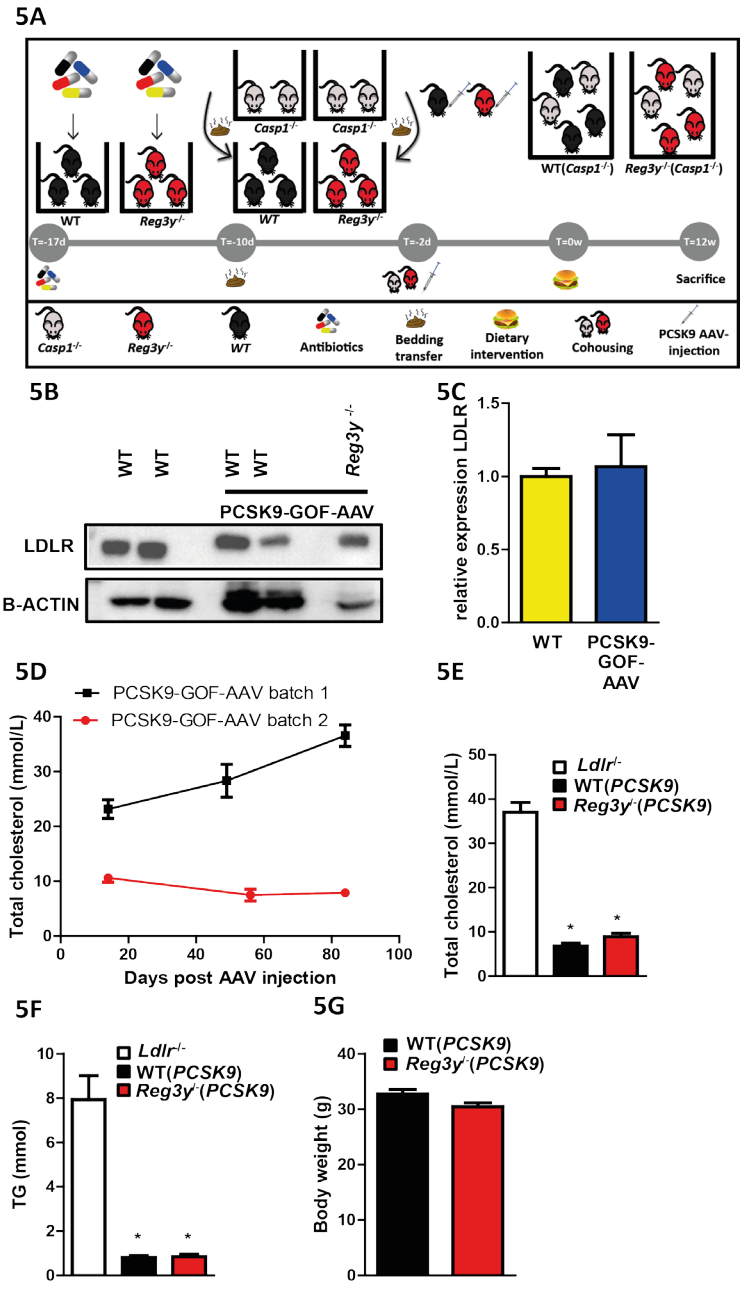


Figure 5 - PCSK9-GOF-AAV injection did not promote hypercholesterolemia following fecal transplantation.

and increased leakage of endotoxins or bacteria into the systemic circulation (Loonen et al., 2013; Martinez-Medina et al., 2014; Wang et al., 2016). However, increased atherogenesis in *Reg3γ*$^{-/-}$(PCSK9) mice was not related to a disruption in intestinal integrity, as we did not observe differences in intestinal inflammation and permeability between *Reg3γ*$^{-/-}$ (PCSK9) and WT(PCSK9) mice (Figure 3). However, the lack of differences in the intestinal permeability in *Reg3γ*$^{-/-}$ mice as measured by the FITC-dextran assay does not exclude translocation of bacteria or endotoxins from the gut lumen into the systemic circulation. Indeed, previous studies have shown that translocation of bacteria from the gut lumen into the mesenteric lymph nodes and liver can occur without significant differences in paracellular transport of bacteria (Wang et al., 2016). In line with this, it has been shown that bacterial transport may occur via the uptake of bacteria by CX3CR1hi expressing mononuclear phagocytes from the lamina propria. These cells can consequently migrate to the mesenteric lymph nodes (Diehl et al., 2013), where the bacteria can reach the systemic circulation via the lymphatic ducts. However, future experiments measuring the presence of bacteria in the circulation and the transport of bacteria via CX3CR1hi mononuclear phagocytes in *Reg3γ*$^{-/-}$ mice are needed to understand whether translocation of bacteria from the gut lumen to systemic circulation contributes to increased atherosclerosis in *Reg3γ*$^{-/-}$ mice. Next to bacteria, endotoxins from the gut lumen can also transport via transcellular routes to the systemic

*Female Ldlr-/- mice aged 12 weeks were exposed to fecal microbiome derived from Casp1-/- or Ldlr-/- mice for 13 weeks while fed a chow diet or a HFC diet. (A) Experimental setup of fecal microbiota transplantation. Female Reg3γ-/- mice or WT littermates were orally gavaged with a cocktail of broad spectrum antibiotics for a period of 10 days to suppress intestinal microbes. This was followed by daily transfer of used bedding material from cages housing Casp1-/- mice to cages housing Reg3-/- mice or WT littermates for 1 week. Reg3γ-/- mice or WT littermates were then injected with 2.0x1010 PCSK9-GOF-AAV and cohoused with Casp1-/- mice in a 3:2 ratio. 2 days post AAV-injection Reg3-/-(Casp1-/-) mice and WT(Casp1-/-) mice were switched to a HFC diet for a period of 13 weeks. (B) Western blot analysis of LDLR in liver. (C) Quantification of western blot analysis. (D) Plasma total cholesterol concentrations over time. (E) Total cholesterol at t=13 weeks (F) Triglycerides at t=13 weeks. (G) Body weight. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired two-tailed Student's t-test.*

circulation. Endotoxins and in particular lipopolysaccharide can be taken up by intestinal epithelial cells where they can bind to chylomicrons, which are abundantly formed during high fat diet feeding (Ghoshal et al., 2009). Consequently the chylomicrons can transport together with the endotoxins to the basolateral side, thereby facilitating the transport of endotoxins from the gut lumen into the systemic circulation (Ghoshal et al., 2009). Endotoxemia was however not involved in the increased susceptibility of *Reg3γ*$^{-/-}$ mice for systemic inflammation in alcoholic steatohepatitis (Wang et al., 2016). Future experiments are needed to understand if endotoxemia contributes to increased atherogenesis in *Reg3γ*$^{-/-}$(PCSK9) mice.

We did not detect a disturbance in microbiota composition between *Reg3γ*$^{-/-}$(PCSK9) and WT(PCSK9) mice (Figure 4). This is in accordance with previous literature showing that *Reg3γ*$^{-/-}$ mice do not harbor a dysbiotic gut microbiota compared to WT mice (Loonen et al., 2013; Vaishnava et al., 2011). To elucidate the protective contribution of the antimicrobial peptide REG3γ against dysbiosis-induced atherosclerosis, we introduced the pro-inflammatory gut microbiota of *Casp1*$^{-/-}$ mice into antibiotic-treated *Reg3γ*$^{-/-}$ and WT mice via a cohousing approach (Figure 5A) (Elinav et al., 2011; Henao-Mejia et al., 2012, Chapter 3). *Reg3γ*$^{-/-}$ (*Casp1*$^{-/-}$) and WT (*Casp1*$^{-/-}$) mice were then injected with a PCSK9-GOF-AAV (Bjorklund et al., 2014) to accelerate atherogenesis. However, total cholesterol levels after 13 weeks of HFC-diet feeding were only mildly increased to 9 mmol/L in contrast to 35 mmol/L in *Reg3γ*$^{-/-}$(PCSK9) or WT(PCSK9) mice (Figure 5D, E). In addition, western blot analysis of LDLR in liver homogenates of *Reg3γ*$^{-/-}$(*Casp1*$^{-/-}$) and WT(*Casp1*$^{-/-}$) mice did not show a downregulation of LDLR (Figure 5B,C). For injection of WT(*Casp1*$^{-/-}$) or *Reg3γ*$^{-/-}$(*Casp1*$^{-/-}$) a new batch of PCSK9-GOF-AAV was generated, a lack of downregulation in LDLR in the liver of these mice indicates that, this batch of PCSK9-GOF-AAV did not significantly promote lysosomal degradation of hepatic LDLR . A limited response to the injection of the PCSK9-GOF-AAV can possibly be explained by a reduction in the proportion of infectious particles generated in the second batch of AAV production.

Overall our study shows that the antimicrobial peptide REG3γ plays a protective role in the development of atherosclerosis. Future experiments need to be conducted to understand how REG3γ protects atherosclerosis development. Especially, investigation of transcellular routes for

transportation of endotoxins and bacteria should be considered. In addition, it is important to repeat the fecal transplantation experiment to understand the protective role of antimicrobial peptides during conditions in which the gut microbiome is disturbed and can further promote atherosclerosis development. A better understanding of the function of antimicrobial peptides and the protection they confer during dysbiosis in the etiology of atherosclerosis may possibly lead to novel targets in protection against atherosclerosis, either by improving antimicrobial peptide production or by specifically targeting specific microbial species invading the intestinal epithelial barrier.

References

Aguilar, E.C., Leonel, A.J., Teixeira, L.G., Silva, A.R., Silva, J.F., Pelaez, J.M.N., Capettini, L.S.A., Lemos, V.S., Santos, R.A.S., and Alvarez-Leite, J.I. (2014). Butyrate impairs atherogenesis by reducing plaque inflammation and vulnerability and decreasing NFκB activation. Nutr. Metab. Cardiovasc. Dis. 24, 606–613.

Bjorklund, M.M., Hollensen, A.K., Hagensen, M.K., Dagnaes-Hansen, F., Christoffersen, C., Mikkelsen, J.G., and Bentzon, J.F. (2014). Induction of Atherosclerosis in Mice and Hamsters Without Germline Genetic Engineering. Circ. Res. 114, 1684–1689.

Brandsma, E., Houben, T., Fu, J., Shiri-Sverdlov, R., and Hofker, M.H. (2015). The immunity-diet-microbiota axis in the development of metabolic syndrome. Curr. Opin. Lipidol. 26, 73–81.

Diehl, G.E., Longman, R.S., Zhang, J.-X., Breart, B., Galan, C., Cuesta, A., Schwab, S.R., and Littman, D.R. (2013). Microbiota restricts trafficking of bacteria to mesenteric lymph nodes by CX(3)CR1(hi) cells. Nature 1–6.

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., et al. (2011). NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. Cell 145, 745–757.

Ghoshal, S., Witta, J., Zhong, J., de Villiers, W., and Eckhardt, E. (2009). Chylomicrons promote intestinal absorption of lipopolysaccharides. J. Lipid Res. 50, 90–97.

Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W.Z., Strowig, T., Thaiss, C.A., Kau, A.L., Eisenbarth, S.C., Jurczak, M.J., et al. (2012). Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482:179–185.

Ishibashi, S., Brown, M.S., Goldstein, J.L., Gerard, R.D., Hammer, R.E., and Herz, J. (1993). Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J. Clin. Invest. 92, 883–893.

Johansson, M.E. V., and Hansson, G.C. (2012). Preservation of Mucus in Histological Sections, Immunostaining of Mucins in Fixed Tissue, and Localization of Bacteria with FISH. In Methods in Molecular Biology (Clifton, N.J.), pp. 229–235.

Joosten, L.A.B., Netea, M.G., Fantuzzi, G., Koenders, M.I., Helsen, M.M.A., Sparrer, H., Pham, C.T., van der Meer, J.W.M., Dinarello, C.A., and van den Berg, W.B. (2009). Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. Arthritis Rheum. 60, 3651–3662.

Koeth, R.A., Wang, Z., Levison, B.S., Buffa, J.A., Org, E., Sheehy, B.T., Britt, E.B., Fu, X., Wu, Y., Li, L., et al. (2013). Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat. Med. 19, 576–585.

Koren, O., Spor, A., Felin, J., Fåk, F., Stombaugh, J., Tremaroli, V., Behre, C.J., Knight, R., Fagerberg, B., Ley, R.E., et al. (2011). Human oral, gut, and plaque microbiota in patients with atherosclerosis. Proc. Natl. Acad. Sci. U. S. A. 108 Suppl 1, 4592–4598.

Krauss, R.M., Eckel, R.H., Howard, B., Appel, L.J., Daniels, S.R., Deckelbaum, R.J., Erdman, J.W., Kris-Etherton, P., Goldberg, I.J., Kotchen, T.A., et al. (2000). AHA Dietary Guidelines: revision 2000: A statement for healthcare professionals from the Nutrition Committee of the American Heart Association. Circulation 102, 2284–2299.

Li, J., Lin, S., Vanhoutte, P.M., Woo, C.W., and Xu, A. (2016). Akkermansia muciniphila protects against atherosclerosis by preventing metabolic endotoxemia-induced inflammation in Apoe-/- Mice. Circulation 133, 2434–2446.

Libby, P., Ridker, P.M., and Hansson, G.K. (2011). Progress and challenges in translating the biology of atherosclerosis. Nature 473, 317–325.

Loonen, L.M., Stolte, E.H., Jaklofsky, M.T., Meijerink, M., Dekker, J., van Baarlen, P., and Wells, J.M. (2013). REG3γ-deficient mice have altered mucus distribution and increased mucosal inflammatory responses to the microbiota and enteric pathogens in the ileum. Mucosal Immunol. 1–9.

Loy, A., Pfann, C., Steinberger, M., Hanson, B., Herp, S., Brugiroux, S., Gomes Neto, J.C., Boekschoten, M. V., Schwab, C., Urich, T., et al. (2017). Lifestyle and Horizontal Gene Transfer-Mediated Evolution of Mucispirillum schaedleri , a Core Member of the Murine Gut Microbiota. mSystems 2, e00171-16.

Lusis, A.J., Mar, R., and Pajukanta, P. (2004). GENETICS OF ATHEROSCLEROSIS. Annu. Rev. Genomics Hum. Genet. 5, 189–218.

Martinez-Medina, M., Denizot, J., Dreux, N., Robin, F., Billard, E., Bonnet, R., Darfeuille-Michaud, A., and Barnich, N. (2014). Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 63, 116–124.

Morgan, X.C., Tickle, T.L., Sokol, H., Gevers, D., Devaney, K.L., Ward, D. V, Reyes, J.A., Shah, S.A., LeLeiko, N., Snapper, S.B., et al. (2012). Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. Genome Biol. 13, R79.

Ott, S.J., El Mokhtari, N.E., Musfeldt, M., Hellmig, S., Freitag, S., Rehman, A., Kühbacher, T., Nikolaus, S., Namsolleck, P., Blaut, M., et al. (2006). Detection of Diverse Bacterial Signatures in Atherosclerotic Lesions of Patients With Coronary Heart Disease. Circulation 113, 929–937.

Su, D., Nie, Y., Zhu, A., Chen, Z., Wu, P., Zhang, L., Luo, M., Sun, Q., Cai, L., Lai, Y., et al. (2016). Vitamin D signaling through induction of paneth cell defensins maintains gut microbiota and improves metabolic disorders and hepatic steatosis in animal models. Front. Physiol. 7, 1–18.

Tang, W.H.W., and Hazen, S.L. (2014). The contributory role of gut microbiota in cardiovascular disease. J. Clin. Invest. 124, 4204–4211.

Vaishnava, S., Yamamoto, M., Severson, K.M., Ruhn, K.A., Yu, X., Koren, O., Ley, R., Wakeland, E.K., and Hooper, L. V. (2011). The Antibacterial Lectin RegIII Promotes the Spatial Segregation of Microbiota and Host in the Intestine. Science (80-.). 334, 255–258.

Veilleux, A., Mayeur, S., Bérubé, J.-C., Beaulieu, J.-F., Tremblay, E., Hould, F.-S., Bossé, Y., Richard, D., and Levy, E. (2015). Altered intestinal functions and increased local inflammation in insulin-resistant obese subjects: a gene-expression profile analysis. BMC Gastroenterol. 15, 119.

Wang, L., Fouts, D.E., Stärkel, P., Hartmann, P., Chen, P., Llorente, C., DePew, J., Moncera, K., Ho, S.B., Brenner, D.A., et al. (2016). Intestinal REG3 Lectins Protect against Alcoholic Steatohepatitis by Reducing Mucosa-Associated Microbiota and Preventing Bacterial Translocation. Cell Host Microbe 19, 227–239.

Yin, K., Tang, S., Yu, X., Tu, G., He, R., Li, J., Xie, D., Gui, Q., Fu, Y., Jiang, Z., et al. (2013). Apolipoprotein A-I inhibits LPS-induced atherosclerosis in ApoE(-/-) mice possibly via activated STAT3-mediated upregulation of tristetraprolin. Acta Pharmacol. Sin. 34, 837–846.

CHAPTER 5

The gut anti-inflammatory agent 5-ASA does not protect against insulin resistance in mice

Zheng Wang[1, 2,] **Eelke Brandsma[2]**, *Ailine G. Lopez Manosalva[2], Niels J. Kloosterhuis[2], Albert Gerding[3], Marten H. Hofker[2]†, Zhijun Bao[1], Bart van de Sluis[2]*, and Debby P.Y. Koonen[2]**

† Deceased
**Equal contribution*

1 Department of Geriatrics and Gastroenterology, Huadong Hospital, Shanghai Medical College, Fudan University, Shanghai Key Laboratory of Clinical Geriatric Medicine, Shanghai 200040, P.R. China
2 University of Groningen, University Medical Center Groningen, Department of Pediatrics, Section Molecular Genetics, Groningen, the Netherlands
3 University of Groningen, University Medical Center Groningen, Department of Pediatrics, Section Systems Medicine of Metabolism & Signaling, Groningen, the Netherlands

Abstract

Objective

5-aminosalicylic acid (5-ASA) is widely used in the treatment of inflammatory bowel diseases (IBD) and was recently shown to improve insulin resistance (IR) in diet-induced obese (DIO) mice through its gut specific anti-inflammatory effect. In addition, cholesterol in western type diets has been suggested to be a driving factor of intestinal inflammation. Therefore, we investigated whether dietary cholesterol can promote intestinal inflammation and attribute to development of type 2 diabetes.

Methods

C57BL/6J mice were fed a low-fat diet (LFD), high fat diet (HFD) or high-fat cholesterol diet (HFCD, 0.25% cholesterol) supplemented with 5-ASA (1500mg/Kg/day) for 12 weeks. Body weight and food intake were recorded weekly. Glucose tolerance test (GTT) was performed on week 11. Fat to lean ratio and gut permeability were measured. Blood, intestines and liver were taken for analysis.

Results

Feeding of HFD or HFCD resulted in obesity and systemic glucose intolerance. HFD or HFCD feeding in comparison with LFD feeding did not affect intestinal permeability as measured by the FITC-dextran assay. Hence, we did not see an effect of administration of the gut anti-inflammatory agent 5-ASA on obesity or systemic glucose intolerance.

Conclusions

Our results indicate that feeding of a HFD or HFCD does not directly affect intestinal barrier function.

Introduction

Obesity is associated with a series of metabolic complications, including dyslipidemia, insulin resistance (IR), type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD). As current preventative and pharmacological therapeutic approaches have had limited success so far, it is evident that new strategies for treating these diseases are urgently needed.

Studies in the past have left little doubt about the involvement of low-grade chronic inflammation in the mechanisms underlying IR and T2D. More recently, several studies have indicated a critical role for the gut in the development of low-grade chronic inflammation in IR and T2D. Consumption of high fat diets promotes intestinal inflammation and triggers disruption of tightjunctions proteins leading to increased intestinal permeability and leakage of endotoxins into the systemic circulation(Cani et al., 2007; Ding et al., 2010; Luck et al., 2015). Leakage of endotoxins into the systemic circulation consequently attributes to low-grade-systemic inflammation and type 2 diabetes. Targeting gut inflammation with 5-aminosalicylic acid (5-ASA) improves systemic metabolic parameters and insulin resistance during high-fat feeding in mice, thereby highlighting the importance of HFD-induced intestinal inflammation in the development of metabolic disease(Luck et al., 2015). Recent studies in zebrafish have indicated that cholesterol in high fat diets is a driving factor for intestinal inflammation (Progatzky et al., 2014). Uptake of dietary cholesterol via NPC1L1 into intestinal epithelial cells leads to inflammasome activation and production of IL-1β leading to the recruitment of neutrophils and macrophages into the gut (Progatzky et al., 2014). Altogether this indicates that dietary cholesterol may contribute to the development of metabolic syndrome by promoting intestinal inflammation leading to leakage of endotoxins into the systemic circulation. To understand the role of dietary cholesterol on intestinal barrier function in the development of the metabolic syndrome, we carried out a set of metabolic experiments in mice fed a low-fat diet (LFD), a high-fat diet (HFD) or a high-fat cholesterol diet (HFCD, 0.25% cholesterol) supplemented with the gut anti-inflammatory agent 5-ASA (1500mg/Kg/day) for 12 weeks. Our data indicate that feeding of a HFD or HFCD for 12 weeks did not lead to increased intestinal permeability, consequently no effects of 5-ASA treatment on development of insulin resistance were observed.

Methods

Mice and treatment

All experiments were performed according to Dutch law and approved by the Ethical Committee for Animal Experiments of the University of Groningen, the Netherlands. Experiments were carried out on male C57BL/6J mice purchased from Charles River (France). Mice were housed separately in Individual Ventilated Cages and maintained on a 12-hour light/12-hour dark cycle with ad libitum access to food and water. Mice were randomized in 6 groups of 10 mice each. Mice were fed either low-fat diet (LFD; 10% kcal fat, Research Diets), LFD 5-ASA, high-fat diet (HFD; 60% kcal fat, 0.02% cholesterol, Research Diets), HFD 5-ASA, HFD supplemented with 0.25% cholesterol (HFC; 60% kcal fat, Research Diets) or HFC 5-ASA starting at 10-12 weeks of age for 12 weeks. Mice were sacrificed using cardiac puncture, and blood, liver and intestines were collected for further analysis.

Compounds and metabolic studies

5-ASA powder (Sigma-Aldrich) was mixed into the LFD, HFD and HFC diets at 1,500 mg/kg/day. All diets were prepared fresh weekly to ensure a consistent 5-ASA dose intake throughout the 12-week study. Body weight and food intake was measured weekly. After 11 weeks of dietary intervention, glucose tolerance test (GTT) was performed as previously described(Gruben et al., 2015), and all GTT were performed with a 2g/kg glucose i.p. injection. Blood samples were collected in EDTA-coated tubes before i.p. injection for determination of insulin levels. Samples were spun at 1000g for 10 min at 4°C and insulin concentrations were measured in plasma by ELISA kit (Alpco Diagnositics, Salem, NH). One day prior to sacrifice body composition was analysed using a Minispec Whole Body Composition Analyser (Bruker).

Gut permeability assays

Gut permeability was measured in unfasted mice at time of sacrifice. Mice received 0.6 mg/g bodyweight of FITC-conjugated dextran (Sigma) by oral gavage and blood was collected via cardiac puncture after 4 hr. The concentration of FITC was determined in plasma by fluorometry at 488 nm as described in Chapter 3.

Quantification of plasma and liver lipids

Plasma triglycerides, total cholesterol and free cholesterol were determined at times of sacrifice using commercially available kits (Triglycerides and total cholesterol: Roche; free cholesterol: FS DiaSys, Holzheim, Germany). To measure hepatic lipid content, lipids were extracted from crushed liver samples using Bligh and Dyer's method(Bligh and Dyer, 1959). Hepatic triglyceride and cholesterol levels were measured using kits that are commercially available (Triglycerides and total cholesterol: Roche; free cholesterol: FS DiaSys).

Quantitative real-time PCR

RNA was isolated using Qiazol reagent, according to the manufacturer's instructions (Roche). cDNA was synthesized using the Transcriptor Universal cDNA Master kit from Roche, according to their instructions (Roche, Mannheim, Germany). We performed quantitative real-time PCR with a 7900HT PCR system (Applied Biosystems) using SYBR Green Master Mix reagent (Roche). Each sample was run in triplicate and normalized to PPIA as housekeeping gene. We calculated relative fold changes in gene expression normalized to PPIA by the ΔΔCT method using the equation 2-ΔΔCT. The results are shown as fold changes compared to the HFC group. Primer sequences are listed in Supplemental table 1.

2.7 Statistical analysis

The data were presented as mean ± SEM unless stated otherwise. Comparisons between groups were performed using one-way ANOVA + tukey HSD post-test unless stated otherwise with $P < 0.05$ considered statistically significant.

Results and Discussion

To understand the role of dietary cholesterol on intestinal health in high-fat diet induced insulin resistance we fed C57BL/6J mice a LFD, HFD, or HFCD with or without the gut anti-inflammatory agent 5-ASA for 12 weeks. Food intake in grams per day was similar between LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD + 5ASA, ensuring that dosage of 5-ASA (1500 mg/Kg/day) was comparable between groups (Figure 1A). Next, we examined the metabolic effects of 5-ASA during feeding of LFD, HFC and HFCD. As expected feeding of HFD or HFCD significantly

increased body weight gain, fat mass and total plasma cholesterol level, whereas triglyceride levels declined (Figure 1B-E). In contrast, these factor were not affected by administration of 5-ASA (Figure 1B-D). Feeding of HFD or HFCD resulted in increased fasting glucose levels and systemic glucose intolerance compared to LFD-fed mice (Figure 2A-B). Treatment of mice with the gut anti-inflammatory agent 5-ASA did however not affect fasting glucose levels or systemic glucose intolerance (Figure 2A, B). This is in contrast to the study by Luck et al, who reported a protective effect of 5-ASA treatment against systemic glucose intolerance.

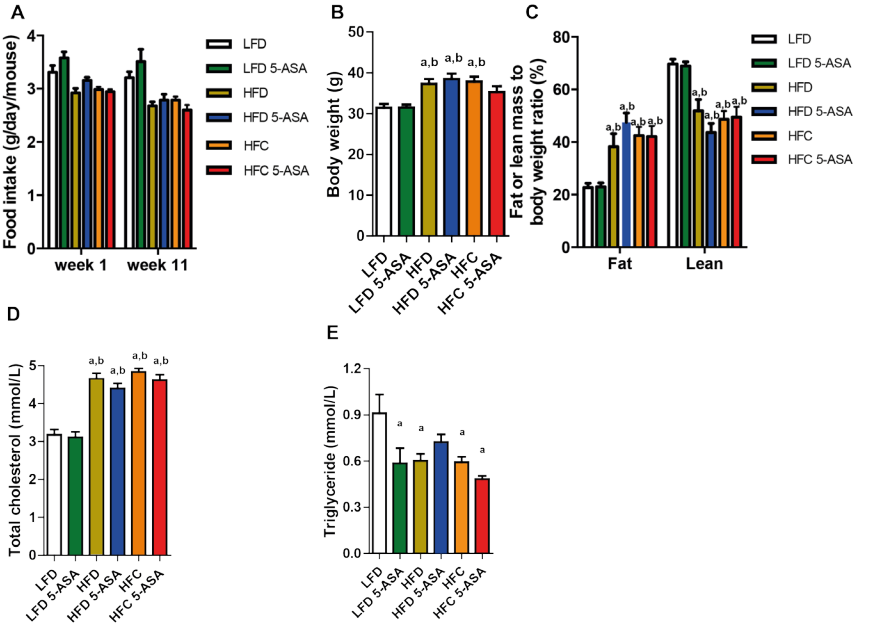


Figure 1 – Feeding of HFD or HFCD diet promotes obesity and hyperlipidemia

(A) Food intake of mice fed LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA at week 1 and week 11 (B) Body weights of mice fed LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA (C) Fat or lean mass, expressed as percentage of body weight, were analyzed after 12 weeks of LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA in C57BL/6J mice over time, starting at 10-12 weeks of age (D) total cholesterol levels in plasma (E) Trigycerides in plasma after 12 weeks of dietary intervention. P<0.05 (a) with respect to LFD, (b) with respect to LFD 5-ASA; by one-way ANOVA + Tukey HSD post-test.

In addition, we examined the effect of 5-ASA against development of hepatic steatosis. Our study shows that feeding of HFD leads to increased storage of triglycerides and total cholesterol in the liver in comparison with LFD (Figure 2C, D). In addition, feeding of a HFCD in comparison with HFD further increases triglyceride and total cholesterol storage in the liver of C57BL/6J mice(Figure 2C, D). Consistently, histological examination of the liver confirmed increased steatosis following feeding of HFCD vs HFD (Figure 2E). Increased storage of triglycerides and total cholesterol in the liver following addition of cholesterol to a HFD diet is in accordance with literature (Wouters et al., 2008). Interestingly, we also observed a significant reduction in storage of triglycerides in the liver following 5-ASA treatment in HFCD-fed mice, but not after HFD-feeding (Figure 2C). A lack of protection against liver steatosis after HFD-feeding is in contrast with the results of Luck et al who reported a reduction in liver steatosis (Luck et al., 2015). To examine whether differences in triglyceride storage in the liver of HFCD-fed mice also resulted in altered inflammatory levels in the liver we performed qPCR analysis in mice receiving HFCD or HFCD + 5-ASA. Treatment with 5-ASA in HFCD-fed mice leading to a reduction in triglyceride storage did not result in decreased liver inflammation (Figure 2F). To understand the discrepancies between our data and the data of Luck et al in the development of systemic glucose intolerance and hepatic steatosis following administration of 5-ASA we examined whether 5-ASA improved intestinal health during HFD and HFCD feeding. We observed that feeding of a HFD or HFCD for 12 weeks in comparison with LFD did not affect intestinal barrier function as measured by the translocation of FITC-dextran from the gut into the systemic circulation (Figure 3). Consequently, we also did not observe a protective effect of 5-ASA (Figure 3). The absence of an effect on intestinal permeability after feeding of HFD or HFCD is highly surprising and in contrast with previous results from our lab (Supplemental figure 1) and many other groups (Cani et al., 2007; Ding et al., 2010; Luck et al., 2015). This surprising finding indicates that dietary fat or cholesterol does not directly affect the gut barrier function in C57Bl/6 mice. Interaction between HFD-feeding and the gut microbiota has been indicated as a factor promoting intestinal inflammation. Feeding of a HFD in conventional mice promoted intestinal inflammation whereas germfree mice were protected (Ding et al., 2010). In addition, feeding of HFD can lead to the expansion of pathobionts

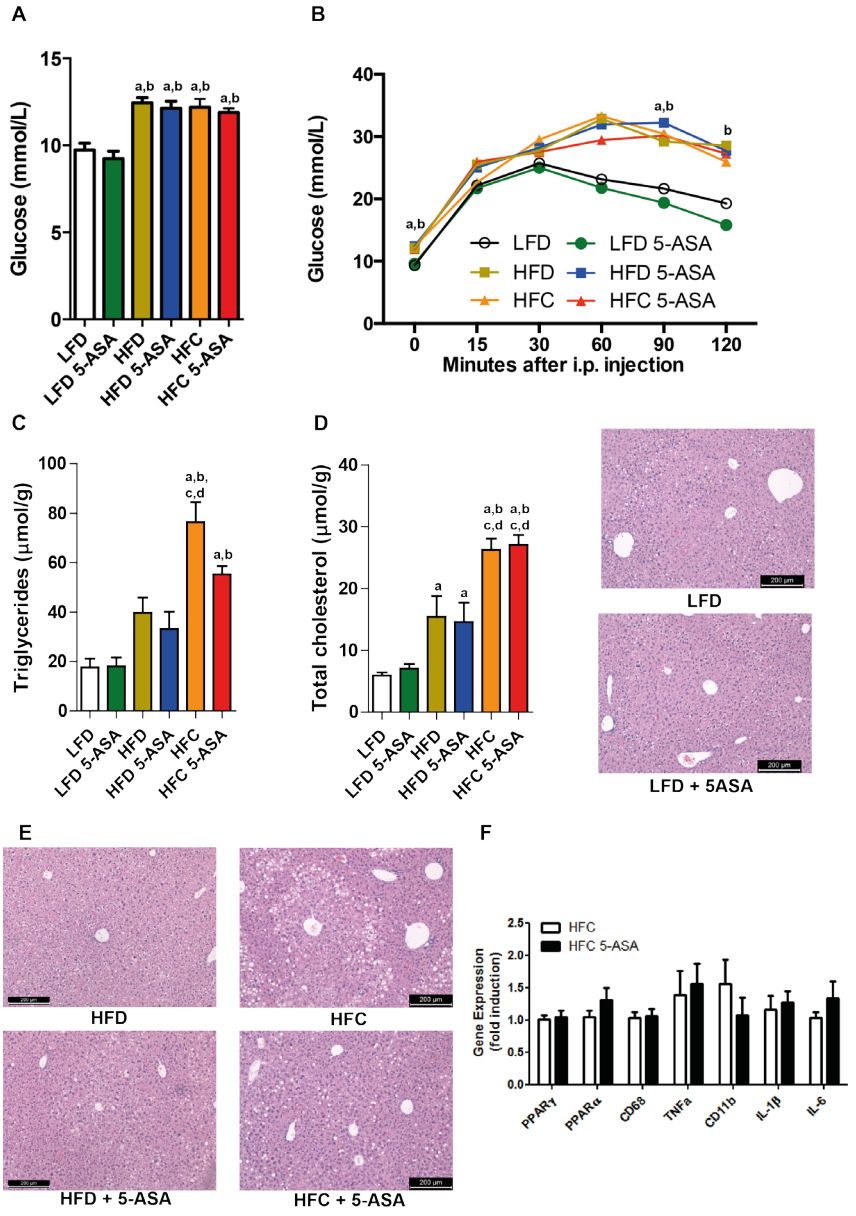


Figure 2 – The gut anti-inflammatory agent 5-ASA does not protect against systemic glucose intolerance or NAFLD

(A) fasted glucose levels after 12 weeks of dietary intervention (B) oral glucose tolerance test (C) triglyceride levels in liver in µmol/g (D) total cholesterol levels

such as Bilophila Wadsworthia and Attaching Invading Eschericia Coli (AIEC), consequently promoting intestinal inflammation (David et al., 2014; Devkota et al., 2012; Martinez-Medina et al., 2014). Thus absence of specific pathobionts in our mice during feeding of HFD or HFCD may prevent development of intestinal inflammation and increased intestinal permeability.

Luck et al showed induction of intestinal inflammation leading to increased intestinal permeability and leakage of endotoxins promoting low-grade systemic inflammation and insulin resistance following feeding of a HFD. HFD- or HFCD-feeding did however not result in decreased barrier function in our study, providing a plausible explanation of why administration of 5-ASA did not result in protection against systemic glucose intolerance and NAFLD in our studies.

Conclusion

In conclusion, our data suggest that consumption of HFD or HFCD does not cause abrogated intestinal barrier function, indicating that dietary fat or cholesterol do not directly affect intestinal barrier function.

Funding

Z.W. is supported by the China Scholarship Council, and grants from Shanghai Key Developing Disciplines Program (No: 2015ZB0501). E.B, M.H. and D.K. are supported by a grant from CardioVasculair Onderzoek Nederland (CVON2012-03).

Conflict of interest

The authors declare no conflict of interest.

in liver in µmol/g (E) hematoxylin and eosin slides of liver (F) inflammatory gene expression in liver of HFCD and HFCD + 5-ASA fed mice.

Data are presented as means ± SEM, n = 10 mice. P<0.05 (a) with respect to LFD, (b) with respect to LFD 5-ASA (c) with respect to HFD (d) with respect to HFD 5-ASA; by one-way ANOVA + Tukey HSD post-test.

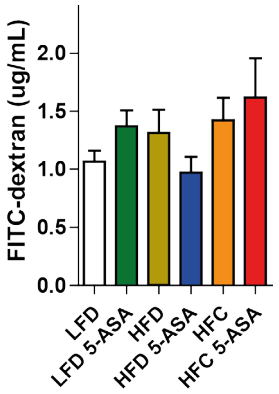
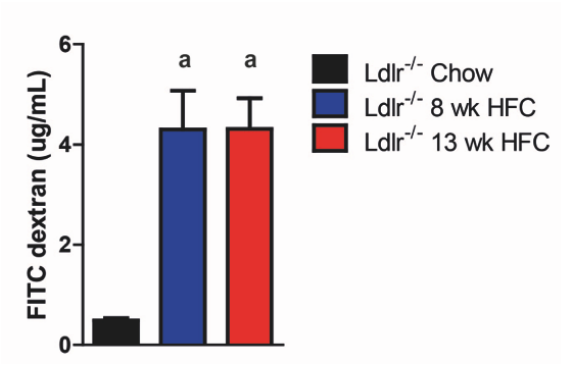


Figure 3 – HFD or HFCD feeding does affect intestinal barrier function in C57BL/6J mice

FITC-dextran in vivo permeability assay in mice fed LFD, LFD + 5-ASA, HFD, HFD + 5-ASA, HFCD and HFCD +5-ASA. Data are presented as means ± SEM, n = 10 mice.



Supplemental figure 1

FITC-dextran in vivo permeability assay in Ldlr-/- mice fed a chow diet, HFC for 8 weeks or HFC diet for 13 weeks. Ldlr-/- chow n=10, 8 weeks HFC n=10 13 weeks HFC n=9. Data are presented as means ± SEM, (a) P<0.05 (a) with respect to Ldlr-/- Chow; by unpaired two-tailed Student's t-test.

References

Bligh, E.G., and Dyer, W.J. (1959). A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. Can. J. Biochem. Physiol. 37, 911–917.

Cani, P.D., Amar, J., Iglesias, M.A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A.M., Fava, F., Tuohy, K.M., Chabo, C., et al. (2007). Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. Diabetes 56, 1761–1772.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A. V., Devlin, A.S., Varma, Y., Fischbach, M.A., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature 505, 559–563.

Devkota, S., Wang, Y., Musch, M.W., Leone, V., Fehlner-Peach, H., Nadimpalli, A., Antonopoulos, D.A., Jabri, B., and Chang, E.B. (2012). Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature 487, 104–108.

Ding, S., Chi, M.M., Scull, B.P., Rigby, R., Schwerbrock, N.M.J., Magness, S., Jobin, C., and Lund, P.K. (2010). High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS One 5, e12191.

Gruben, N., Funke, A., Kloosterhuis, N.J., Schreurs, M., Sheedfar, F., Havinga, R., Houten, S.M., Shiri-Sverdlov, R., van de Sluis, B., Kuivenhoven, J.A., et al. (2015). Cholesterol-induced hepatic inflammation does not underlie the predisposition to insulin resistance in dyslipidemic female LDL receptor knockout mice. J. Diabetes Res. 2015, 956854.

Luck, H., Tsai, S., Chung, J., Clemente-Casares, X., Ghazarian, M., Revelo, X.S., Lei, H., Luk, C.T., Shi, S.Y., Surendra, A., et al. (2015). Regulation of Obesity-Related Insulin Resistance with Gut Anti-inflammatory Agents. Cell Metab. 21, 527–542.

Martinez-Medina, M., Denizot, J., Dreux, N., Robin, F., Billard, E., Bonnet, R., Darfeuille-Michaud, A., and Barnich, N. (2014). Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 63, 116–124.

Progatzky, F., Sangha, N.J., Yoshida, N., McBrien, M., Cheung, J., Shia, A., Scott, J., Marchesi, J.R., Lamb, J.R., Bugeon, L., et al. (2014). Dietary cholesterol directly induces acute inflammasome-dependent intestinal inflammation. Nat. Commun. 5, 5864.

Wouters, K., van Gorp, P.J., Bieghs, V., Gijbels, M.J., Duimel, H., Lütjohann, D., Kerksiek, A., van Kruchten, R., Maeda, N., Staels, B., et al. (2008). Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. Hepatology 48, 474–486.

CHAPTER 6

Discussion

Introduction

Treatment for cardiovascular disease (CVD) has improved markedly through protection against hyperlipidemia and cardiovascular death by statins and PCSK9-inhibitors. Combining statins and PCSK9-inhibitors successfully reduces the number of cardiovascular events by 50%. This reduction in cardiovascular events is one of the largest success stories in modern medicine (Hansson et al, Circulation, 2017). However, the flip side of the coin is that there is 50% of cardiovascular events remaining, indicating that factors independent of hyperlipidemia also strongly affect the etiology of cardiovascular disease. Over the years an important role for inflammation in the etiology of atherosclerosis has been established. An increase in pro-inflammatory cytokines (e.g IL-1β, IFN-γ) as well as an increase in the number of circulating leukocytes is involved in atherogenesis. Furthermore neutrophils, monocytes and T-cells can infiltrate the atherosclerotic plaque and contribute to atherogenesis by the formation of foam cells and production of reactive oxygen species, increasing the risk of thrombotic events (Libby et al., 2011, Chapter 1)**.** Until recently our understanding of the importance of inflammation in the etiology of atherosclerosis rested on associative studies in human and mechanistic studies in mice, however, recently the CANTOS-trial showed that administration of Canakinumab, an IL-1β antibody, reduced the number of cardiovascular events by 15%. Importantly, all patients in this study received statin treatment and blood lipids were in the normolipidemic range. This indicates that inflammation is involved in the etiology of atherosclerosis independently of hyperlipidemia. Although directly targeting inflammation by Canakinumab successfully reduced the number of cardiovascular events, there was also an increase in the number of fatal infections and sepsis. This is an intrinsic property of anti-inflammatory drugs, therefore understanding the factors that contribute to systemic inflammation during atherogenesis is of utmost importance.

Gut microbiota-diet-immune interactions in atherogenesis

Systemic inflammation and atherosclerosis have been linked to changes in gut microbiota composition (Jie et al., 2017; Karlsson et al., 2012; Schirmer et al., 2016). The gut microbiota is strongly affected by dietary intake and the intestinal immune barrier plays an important role by controlling the gut microbiota (Brandsma et al., 2015). Consumption of a

high fat cholesterol rich diet (HFC) leads to an expansion of pathobionts and to a reduction in the production of beneficial metabolites such as short-chain fatty acids (SCFA) by the microbiota (Brandsma et al., 2015). This can lead to an increase in intestinal permeability and translocation of bacteria or bacterial components into the circulation, where these can trigger an inflammatory response (discussed in **Chapter 2)**. In addition, the gut microbiota is also influenced by the intestinal immune system (Caricilli et al., 2011; Henao-Mejia et al., 2012; Vijay-Kumar et al., 2010). The intestinal immune system needs to balance between tolerating commensal bacteria, while eliminating pathogens. The first line of defense is formed by a physical-chemical layer formed by the production of mucus and antimicrobial peptides. This layer prevents infiltration of bacteria into the intestinal epithelial barrier and is important to prevent translocation of bacteria from the gut into the systemic circulation (Brandsma et al., 2015; Muniz et al., 2012; Sperandio et al., 2015). Furthermore, pattern recognition receptors (PRRs) can recognize bacterial patterns, promoting signaling to the immune system and promoting clearance of pathogens(Hemmi and Akira, 2005). Dysfunction of several PRRs leads to an altered microbiota composition and this has been linked to decreased immune barrier function, increased translocation of endotoxins into the circulation and promotes the metabolic syndrome (Henao-Mejia et al., 2012, **Chapter 2**). Thus, the gut microbiota is influenced by dietary intake and the intestinal immune system and the gut microbiota has been linked to systemic inflammation and atherosclerosis. It is however unclear whether alterations in *microbiota composition* or *localization* can directly contribute to systemic inflammation and atherogenesis and how these processes are affected by the intestinal immune barrier and diet. Therefore, the aim of this thesis was to understand how the interaction between the diet, gut microbiota and intestinal immune barrier contribute to systemic inflammation and atherosclerosis. To better understand these complex processes, we have manipulated the gut microbiota (**Chapter 3**) and the intestinal immune barrier (**Chapter 4**) and studied the effects on atherogenesis. In addition, we assessed the role of dietary cholesterol in Western type diets on gut health in the context of the metabolic syndrome by feeding mice western type diets combined with the gut anti-inflammatory agent 5-ASA (**Chapter 5**).

Main findings of this thesis

In **Chapter 3** we show that transplantation of the pro-inflammatory *Casp1*$^{-/-}$ microbiota into atherosclerosis prone *Ldlr*$^{-/-}$ mice promotes atherogenesis and systemic inflammation. These findings are possibly caused by a reduced capacity of the gut microbiome to produce anti-inflammatory SCFAs, hence affecting systemic inflammation and atherosclerosis.
In **Chapter 4** we show a protective role for the antimicrobial peptide *REG3*γ in the development of atherosclerosis. The antimicrobial peptide *REG3*γ is important to prevent infiltration of the microbiota into the intestinal epithelial barrier. Thus, our study indicates that the intestinal immune barrier plays an important role in the protection against atherogenesis.
In **Chapter 5** we show that increased levels of cholesterol in the diet does not affect intestinal barrier function and T2D in C57BL/6 mice.

The microbiota and cardiovascular disease

The gut microbiota has been linked to cardiovascular disease (CVD) by several observational studies (Emoto et al., 2016; Jie et al., 2017; Karlsson et al., 2012). These studies have seen an increased abundance in the taxonomies *Collinsella, Ruminococcus Gnavus, Escherichia Coli, Klebsiella* and *Enterobacter aerogenes* in CVD patients. Whereas the SCFA-producing bacteria *Eubacterium* and *Roseburia* were decreased (Karlsson et al., 2012). Furthermore, the gut microbiota has been linked to risk factors of CVD, such as blood lipids (TG and HDL) (Fu et al., 2015). However, these observational studies only provide associative evidence that link the gut microbiota to CVD. In addition, the number of studies showing a causal role for the gut microbiota are limited (Li et al., 2016; Wang et al., 2011) and are mostly confined to the understanding of the importance of trimethylamine-oxide (TMAO) in atherogenesis (Gregory et al., 2015; Senthong et al., 2016; Tang et al., 2013; Wang et al., 2011). Choline, phosphatidylcholine, L-Carnitine and other TMA-containing structures are converted by the CutC cluster of the microbiota into TMA (Craciun and Balskus, 2012). TMA is subsequently taken up into the systemic circulation where it is oxidized by Flavin Monooxygenase 3 (FMO3) in the liver leading to formation of TMAO. Increased levels of TMAO augment the development of aortic atherosclerotic plaques (Figure 1a) (Wang et al., 2011). Production of TMA from choline is dependent on the taxonomies; *RF39, Erysipelotrichaceae, Coriobacteriaceae,*

Allobaculum and *Prevotella* (Gregory et al., 2015). These studies have further been confirmed in humans, where gut microbiota dependent production of TMA and consequent oxidation to TMAO promotes atherosclerosis (Tang et al., 2013). However, in chapter 3 we provide a novel, alternative mechanism by which the microbiome may contribute to atherogenesis, independent from TMAO and lipids. We show that introduction of the pro-inflammatory gut microbiome of *Caspase1*$^{-/-}$ mice into *Ldlr*$^{-/-}$ mice increased systemic inflammation and accelerated atherosclerosis. We observed an increase in the inflammatory cytokines IL-1β, Il-2 and IFN-γ as well as an increase in circulating monocytes and neutrophils. This indicates that alterations in the gut microbiota promote systemic inflammation and atherogenesis (Figure 1b). This is an intriguing finding since recent studies have indicated that systemic inflammation is causally involved in CVD independent of hyperlipidemia (Hansson, 2017; Ridker et al., 2017). Nevertheless, we cannot conclude from our study whether the increased atherogenesis following dysbiosis is a consequence of the increased systemic inflammation. Future experiments combining microbiota transplantations with anti-inflammatory drugs are needed to confirm the dependence of increased atherogenesis following dysbiosis on increased systemic inflammation.

SCFA regulators of inflammation

In chapter 3, we also observed a reduction in the SCFA producing taxonomies *Akkermansia, Christensenellaceae* and *Odoribacter* in *Ldlr*$^{-/-}$ mice co-housed with *Casp1*$^{-/-}$ mice. This was accompanied by a reduced cumulative concentration of the anti-inflammatory SCFAs acetate, butyrate and propionate in the cecum of *Ldlr*$^{-/-}$*(Casp1*$^{-/-}$*)* mice. In addition to influencing metabolic processes (**Chapter 2**), SCFAs are involved in the regulation of the immune system and have previously been shown to reduce inflammation (Cox et al., 2009; Tedelind et al., 2007; Vinolo et al., 2011). SCFA affect the immune system locally, by stimulating the production of antimicrobial peptides and IL-18 in intestinal epithelial cells (Corrêa-Oliveira et al., 2016). Production of antimicrobial peptides is important to prevent infiltration of the microbiota into the intestinal epithelial barrier, whereas IL-18 production stimulates the proliferation of intestinal epithelial cells thereby promoting maintenance and repair of the intestinal epithelial barrier (Lewis and Heaton, 1997; Nemoto et

al., 2012; Siegmund, 2010; Tiihonen et al., 2010). Following uptake of SCFAs into the systemic circulation, SCFAs can also affect the systemic immune system. Administration of SCFAs to macrophages following stimulation by the pro-inflammatory stimulus LPS leads to a reduction in the production of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Liu et al., 2012; Singh et al., 2014; Tunaru et al., 2003). Furthermore, butyrate affects antigen presentation by dendritic cells, thereby inhibiting the stimulation of naïve T-cells and hence preventing activation of the adaptive immune response (Millard et al., 2002). Interestingly, butyrate not only inhibits the stimulation of naïve T-cells by dendritic cells, but also skews the differentiation of naïve T-cells from pro-inflammatory IFN-γ producing T-helper 1 cells into more anti-inflammatory regulatory T-cells (Treg) (Gurav et al., 2015). Stimulation of Tregs by butyrate is further supported by *in vivo* evidence, where administration of butyrate in antibiotic-treated mice increases the number of peripheral Tregs. Acetate has also been reported to have anti-inflammatory properties (Cox et al., 2009; Tedelind et al., 2007) but is not as potent as butyrate in reducing pro-inflammatory cytokines (Vinolo et al., 2011). Acetate promotes production of the anti-inflammatory cytokine IL-10 under steady state conditions and promotes clearance of pathogens during infection by promoting development of effector T-cells. The regulation of T-cell development by acetate are dependent on direct histone deaceylase inhibitor activity. Inhibition of HDAC activity by acetate causes acetylation of p70 S6 kinase and phosphorylation of rS6 which regulate the mTOR pathway and this consequently effects generation of Th1, Th17 and IL-10+ T-cells (Koh et al., 2016; Park et al., 2015). Thus, SCFAs seem to reduce inflammation by affecting pro-inflammatory cytokine production, antigen presentation and skewing of T-cell differentiation to Tregs during steady state and can skew T-helper cell development towards Th1 and Th17 cells during infection(Park et al., 2015). This suggest that the reduced production of SCFAs by the gut microbiota of *Casp1*$^{-/-}$ mice may augment systemic inflammation and thereby promote atherogenesis (Fig 1B). In line with this, oral administration of butyrate has been shown to slow the progression of atherosclerosis in *ApoE*$^{-/-}$ mice by attenuating the adhesion and migration of macrophages and decreasing pro-inflammatory cytokines in atherosclerotic plaques (Aguilar et al., 2014). The link between butyrate, inflammation and atherosclerosis is further

supported in humans, where the capacity to produce butyrate by the microbiota is negatively correlated with C-reactive protein concentrations in patients with atherosclerosis (Karlsson et al., 2012). In addition, our study now also reveals a possible role for acetate as protective factor in systemic inflammation and atherogenesis, since the reduced SCFA cecum levels were mainly driven by a significant reduction in acetate. However, future experiments are needed to 1) validate whether reduced SCFA production by the gut microbiota promotes systemic inflammation and atherogenesis and 2) to specify which SCFA or combination of SCFAs are responsible for these effects. A better understanding of SCFA production by bacteria from the gut microbiota in the development of atherosclerosis can possibly reduce the risk for atherosclerosis by administration of specific bacterial strains, dietary fibers or direct administration of SCFA.

Intestinal health and cardiometabolic disease

The intestinal immune barrier plays an important role in controlling the microbiota composition, balancing between tolerance for commensal bacteria and an adequate immune response against pathobionts (Chapter 2). The first line of defense is formed by a physical-chemical layer formed by the production of mucus and antimicrobial peptides (ref). This layer prevents infiltration of bacteria into the intestinal epithelial barrier and is important to prevent translocation of bacteria from the gut into the systemic circulation (**Chapter 2**). In **chapter 4** we investigated whether the mucosal immune system is an additional player in the development of atherogenesis. To do so, we studied the role of the antimicrobial peptide *Reg3γ* in atherogenesis and found an increased atherosclerotic lesion size in *Reg3γ*$^{-/-}$ mice fed a HFC diet. This suggests that increased infiltration of the intestinal epithelial barrier by bacteria from the gut contributes to atherogenesis. Thus, in addition to gut microbiota composition we now also show that bacterial localization in the gut is important during atherogenesis giving further insight into the mechanism by which the microbiota can contribute to cardiovascular disease (Figure 1c). Interestingly, Surana et al identified the bacteria *Ruminococcus Gnavus* and *Lactobacillus Reuteri* as inducers of *Reg3γ* expression, administration of these bacteria in mice increased *Reg3γ* expression (Surana and Kasper, 2017). Therefore, it would be interesting to explore whether administration of these bacteria is protective against atherosclerosis by increasing the

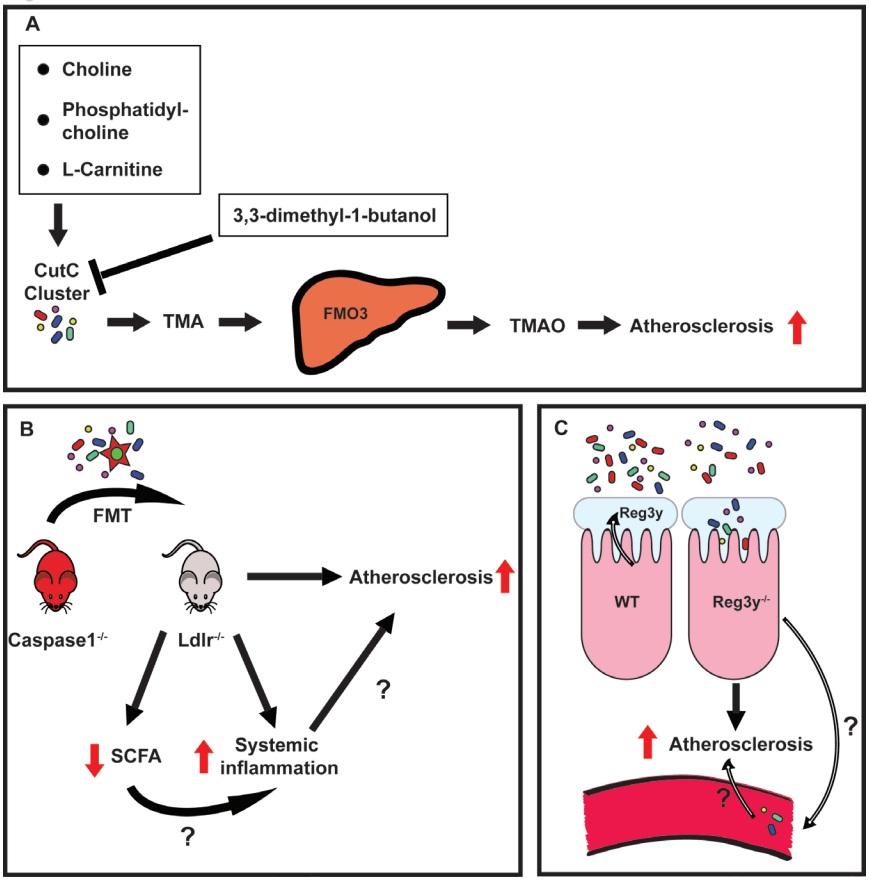


Figure 1. The involvement of the gut microbiota in atherogenesis.

A) *Choline, phosphatidylcholine and L-carnitine can be converted by the CutC cluster in bacteria of the microbiota into TMA. TMA is consequently taken up into the circulation where TMA is oxidized in the liver by FMO3 resulting into the formation of TMAO. Increased levels of TMAO promotes atherogenesis. 3,3dimethyl-1-butanol (DMB) can inhibit the formation of TMA by bacteria in the gut and is protective against atherogenesis.*

B) *Transplanting the microbiota of Caspase1-/- mice into Ldlr-/- mice promotes systemic inflammation and atherogenesis and leads to a decreased production of the anti-inflammatory SCFA. Future research needs to establish whether enhanced atherogenesis is indeed dependent on 1) a reduction in SCFA, 2) whether this results in increased systemic inflammation and 3) whether and increase in systemic inflammation results into increased atherogenesis.*

levels of REG3γ.

Interestingly, western type diets which are important in the onset of cardiovascular disease affect the intestinal immune barrier, leading to a reduction in antimicrobial peptide production, low-grade intestinal inflammation and increased intestinal permeability facilitating the leakage of pro-inflammatory endotoxins into the circulation(Cani et al., 2008; Luck et al., 2015; Wang et al., 2014). In **Chapter 5** we investigated the effects of a western type diet on the immune barrier and how this effects the metabolic syndrome by combining the feeding of a high fat diet (HFD) or HFC with the anti-inflammatory agent 5-ASA in C57BL/6 mice. 5-ASA inhibits intestinal inflammation and prevents increased intestinal permeability following consumption of HFD (Luck et al., 2015). We did not detect differences in intestinal permeability between mice fed a LFD, HFD or HFC indicating that dietary fat or cholesterol does not affect gut barrier function in C57Bl/6 mice. The absence of an effect on intestinal permeability following administration of HFD or HFC is highly surprising and in contrast with our own results (**Chapter 3**) and many other groups in the field (Cani et al., 2008; Ding et al., 2010; Luck et al., 2015). Previous studies have reported the expansion of pathobionts following feeding of western type diets (David et al., 2014; Devkota et al., 2012; Martinez-Medina et al., 2014). The discrepancy between our study **(Chapter 5)** and other studies in the field may indicate that effects of western type diets on intestinal inflammation may be dependent on the presence of pathobionts in the gut microbiota of the host. Future experiments should investigate under which circumstances western type diets can disrupt the intestinal epithelial barrier to understand when and how intestinal inflammation could be targeted for therapy in metabolic diseases.

C) *Reg3γ-/- mice have increased infiltration of bacteria from the microbiota into the intestinal epithelial barrier and Reg3γ-/- display increased susceptibility for the development of atherosclerosis. Future research needs to establish, whether increase susceptibility for atherosclerosis development is a consequence of translocation of bacteria or endotoxins into the circulation via a yet unknown mechanism.*

Understanding the complex interactions of the gut microbiota and the host

The main discovery that has set the stage for microbiota research was done by the group of Jeffrey Gordon in 2006 (Turnbaugh et al., 2006). In this hallmark experiment Gordon's group discovered that transplanting the gut microbiota of obese mice into lean germfree mice promoted adiposity. This key experiment has sparked the interest of the scientific community and set the stage for a quickly developing research field. Due to the relative short timespan since the "start" of the microbiota field, experimental design and experimental techniques are still under development. The design and choice of model for animal experiments in microbiota research needs careful consideration. There are however some ground rules that apply to all models for the design of animal experiments in general and microbiota studies particular. One of the most important factors influencing microbiota composition is the diet (Brandsma et al). Although macromolecular content is well controlled in most studies, the source of fat, protein and fiber content in commercially available diets is not constant and dependent on price fluctuations in the market (Ericsson and Franklin, 2015). As diets generally contain microbial components, diets used for microbiota studies should be irradiated to provide sterilization of the diet to prevent introduction of new bacteria to the gut microbiota of the host (Ericsson and Franklin, 2015). Thus, it is important to select a vendor for the production of diets that provides irradiated diets with a constant composition to exclude confounding factors and to enable reproducibility of results. However, also the choice of mouse vendor can strongly influence the experimental study outcome. Indeed, several studies have detected differences in gut microbiota composition of mice purchased from different vendors (Denning et al., 2011; Hufeldt et al., 2010; Ivanov et al., 2008). Ivanov et. al. found that mice derived from Taconic farms harbor Segmented Filamentous Bacteria (SFB), whereas these are absent in the same mouse strain from Jackson laboratories. Interestingly SFB stimulate the production of serum amyloid A in the terminal ileum, which stimulates dendritic cells in the lamina propria to promote differentiation of Th17 cells(Ivanov et al., 2009). This induction of Th17-cells by SFB promoted resistance against infection with Citrobacter Rodentium (Ivanov et al., 2009). Therefore, it is important to consider the source of mice carefully. Ideally vendors of mice should keep

track of the microbiota composition of mouse lines over time and publish this data on their websites. In addition, specific mouse lines produced by research groups should be donated to a centralized facility following publication, to prevent variabilities in microbiota composition. This will be important for reproducibility of mouse studies in general and microbiota studies in particular.

Furthermore, housing conditions are important to consider for carefully controlled microbiota studies. Traditionally mice are kept in open cages where they are exposed to the environment. However, the environment in an animal facility is hard to control from the perspective of micro-organisms since researchers, cleaning staff and animal care takers frequently enter the animal rooms where the mice are housed and many different mouse lines are typically kept in the same room. To control for these factors mice should be housed in cages that protects against introduction of micro-organisms from the environment, such as IVC cages or flexible film isolators. Individually filtered cages (IVC) are filter top cages placed in a special rack that filters the air that enters each individual cage and prevents exposure of mice to the environment in the animal facility, thereby limiting the unwanted factors that can affect the experimental outcome. Alternatively, mice can be placed in cages within flexible film isolators, flexible film isolators are a completely sterile environment in which cages with mice can be placed. All material introduced into these isolators is autoclaved, these isolators are typically used for germfree mouse experiments (discussed later).

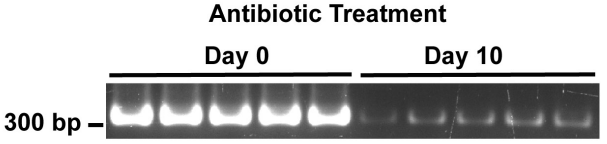


Figure 2. Total bacterial content following antibiotic treatment in *Ldlr*$^{-/-}$ mice

Total bacterial content in feces from Ldlr$^{-/-}$ *mice treated with broad spectrum antibiotics (Metrodinazole, Ampicillin, Neomycin, Vancomycin) for 10 days. The PCR bands from 5 representative mice are shown.*

Mouse models for microbiota research

To investigate the involvement of the microbiota in host physiology and the etiology of disease, several experimental model and designs are available that take into consideration the status of the microbiota of the host. The following mouse models have been used in the microbiota field and have all served their purpose:

Complex microbiota; conventional animals harbor a complex microbiota composition resembling the natural situation

Defined microbiota; animals harboring a microbiota composition of selected bacterial species with a limited complexity, the most well-known example is the altered Schaedler flora containing 8 different bacterial strains.

Mono-associated animals; animals associated with one specific bacterial strains

Germfree animals: animals that are free of all micro-organisms including bacteria, viruses and fungi.

Treating conventional mice with antibiotics as a tool to prove dependence of a phenotype on the gut microbiota is an easy and practical approach to get insight into the involvement of the gut microbiota, an approach that has been successfully applied over time (Elinav et al., 2011; Ichinohe et al., 2011; Rakoff-Nahoum et al., 2004). Administration of broad-spectrum antibiotics can reduce bacterial presence in the gut to 1% of the original (Hill et al., 2010; Rakoff-Nahoum et al., 2004), thereby involvement of the gut microbiota in a phenotype can be investigated. In chapter 3 we also made use of broad-spectrum antibiotics to reduce bacterial presence in the gut. We collected fecal samples before and after the 10-day antibiotic treatment to confirm the effectiveness of the antibiotic treatment. Consistent with previous studies (Hill et al., 2010; Rakoff-Nahoum et al., 2004), total bacterial content was dramatically reduced in *Ldlr*$^{-/-}$ mice that received antibiotics for 10 days (Fig. 2).
It is however noteworthy to realize that this approach relies on the assumption that the registered change in the phenotype is a consequence

of the reduction of the microbiota and not of the antibiotics on the host. Interestingly, Han et al have reported that antibiotics may also directly affect the phenotype of mice independent of the gut microbiota. Disrupting TRAF6 signaling in dendritic cells (TRAF6-DC-/-) exacerbates intestinal inflammation, and this effect was ameliorated after administration of antibiotics (Han et al., 2015). However, administration of antibiotics to germfree TRAF6-DC-/- mice was also shown to ameliorate intestinal inflammation, indicating that the effects of antibiotics were independent of the gut microbiota (Han et al., 2015). Although this is most likely an exception to the rule, the possibility of direct effects of antibiotics should be taken into account during study design and interpretation of the study results.

In addition to reducing the presence of the gut microbiota via administration of antibiotics, germfree mice have been used extensively in the field to investigate the involvement of the microbiota in a phenotype (Bäckhed et al., 2004; Taurog et al., 1994; Turnbaugh et al., 2006). In this experimental design germfree mice are compared with conventional mice. Differences between the phenotype of germfree and conventional mice indicates the involvement of the microbiota. The advantage of germfree mice with respect to conventional mice treated with broad-spectrum antibiotics is that all bacteria are absent, whereas combining broad-spectrum antibiotics strongly reduces the number of bacteria in the gut but does not remove all bacteria (Figure 2). However, there is a lot of debate about the suitability of germfree mice as a physiological model. Bacteria are essential for the development of the immune system and germfree mice display an underdeveloped immune system (Atarashi et al., 2011; Gaboriau-Routhiau et al., 2009; Helgeland et al., 1996; Ivanov et al., 2009). In addition, intestinal epithelial gene expression is affected (Chowdhury et al., 2007; Hooper et al., 2001), intestinal turnover slowed down (Savage et al., 1981) and germfree mice need specialized diets. Specialized diets are necessary to provide the microbiota dependent Vitamin K and to ensure that the diet is low in fiber content. Due to the absence of bacteria in the gut, fibers cannot be degraded, leading to enlarged ceca and obstipation of germfree mice, to prevent these type of problems specialized diets for germfree mice have been developed. Another important limitation of conducting germfree experiments is the complexity of deriving specific mouse lines to a germfree status.

To transform a specific mouse line into a germfree status, surrogate germfree mice can be purchased and placed in flexible film isolators for maintenance of the germfree status. To rederive a mouse line of interest into a germfree status, mice need to be time mated together with the surrogate germfree mice that will serve as foster mothers. The foster mothers are timed to have pups 1-2 days before the strain of interest, pups from the fosters will be removed and pups from the mouse strain of interest will be removed via hysterectomy and transferred sterilely to the germfree foster mothers. During production and maintenance of the new germfree mouse line, the germfree status needs to be monitored regularly to ensure germfree status. A surplus of breeding pairs is needed to achieve sufficient mice for an experiment, due to variability in timed mating and cannibalism of foster mothers. Cannibalism can be reduced by minimizing handling of animals and reducing activity in the room to an absolute minimum (Carter et al., 2002). Altogether, germfree mice are interesting as a model because there is no influence of other micro-organisms, but rederiving mice into a germfree status is a tedious process and the germfree status itself has a large effect on physiology of mice.

Fecal microbiota transplantation

Next to ablating the microbiota composition, introduction of the microbiota into mice is an important tool to investigate the involvement of the involvement of the microbiota. A commonly used and probably the simplest approach to investigate causality of the microbiota in disease development is cohousing of diseased mice together with healthy mice. Since mice are coprophagic microbiota transfer occurs naturally via consumption of fecal pellets by mice. This approach has been used to show the involvement of the microbiota in obesity as well as NAFLD (Henao-Mejia et al., 2012; Ridaura et al., 2013). Ridaura et al reported a protective effect against obesity after cohousing obese mice together with lean mice, this protective effect was dependent on the transfer of Christensenellaceae from lean mice to obese mice (Ridaura et al., 2013). On the other hand also exacerbation of phenotypes has been registered after cohousing animals, Henao-Mejia et al have reported exacerbation of NAFLD of WT mice after cohousing with Nlrp3-/- mice (Henao-Mejia et al., 2012). Furthermore, a mixed phenotype has been reported, cohousing the same mouse strain from 2 vendors with low

and high susceptibility for acute liver injury resulted in an intermediate phenotype. (Celaj et al., 2014). In addition, we show increased systemic inflammation and enhanced atherogenesis after cohousing *Ldlr-/-* mice with *Casp1-/-* mice harboring a pro-inflammatory microbiota (chapter 3). Thus, cohousing conventional mice is a useful approach to investigate the involvement of the gut microbiota in disease, however the direction and effectiveness of microbiota transfer cannot be controlled and is likely dependent on the stability of the ecosystem in the gut of the mouse strains. To control the direction and effectiveness of fecal microbiota transplantation (FMT) it is important to reduce the microbiota composition of the recipient mice. This can be achieved by making use of mice with altered schaedler flora, germfree mice, treating recipient mice with a mixture of broad-spectrum antibiotics or via cross-fostering. Cross-fostering is achieved via timed matings, where pups that receive an alternate microbiota composition (microbiota recipients) are placed with a foster mother that serves as the microbiota donor. Development of the microbiota start during delivery therefore it is important to transfer the pups with the foster mother directly after birth. Cross-fostering is an effective method (Couturier-Maillard et al., 2013; Fuhrer et al., 2010; Garrett et al., 2007) to transfer the microbiota composition and test the physiological consequences of the altered microbiota composition. As with production of germfree mice, cross-fostering is labour intensive and requires a surplus of breeding pairs. It is however not necessary to have a germfree facility and experiments can be conducted in IVC-cages present in most animal facilities. In addition, cross-fostering is the natural way in which the gut microbiota develops, development of the immune system occurs normally and there is no limitation in types of diets that can be fed to these mice. To maintain the introduced microbiota composition over time, mice should be placed together with donor mice during the entire course of the experiment. Next to cross-fostering, the microbiota can also be transplanted into germfree mice. An important advantage of microbiota transplantations into germfree mice with respect to cross-fostering is the ability to transplant the microbiota composition at different time points, thereby giving insight into the effect of the microbiota during immune development. The microbiota can be transplanted into germfree mice either via oral gavage or by cohousing germfree mice together with the fecal donors. Oral gavage of the gut microbiota is a well-controlled

process, but does increase the stress of animals during experiments due to regular gavages, whereas cohousing leads to natural ingestion of the microbiota but is less well controlled. These approaches for FMT can also be applied to mice in which the microbiota has been reduced with broad-spectrum antibiotics (chapter 3. Figure 2). In case of cohousing, a washout period should be taken into account. Recipients of FMT will have residual antibiotics present in their feces, therefore consumption of fecal pellets of FMT recipients by the FMT donors could affect the microbiota composition and should be prevented. This can be achieved by transferring the bedding of the FMT donors into the cage of the FMT recipients for 1 week following the treatment with antibiotics, after this washout period FMT recipients and FMT donors can be housed together to maintain the transplanted microbiota composition for the rest of the experiment (Chapter 3). Altogether, microbiota experiments should be conducted in a facility in which mice are protected against influences of the environment via IVC-cages or flexible film isolators and food should be irradiated and purchased from a vendor with standardized sources for the macromolecular composition of the food. FMT can be performed by transplanting the microbiota of FMT donors via cross-fostering, treating FMT recipients with broad-spectrum antibiotics or using germfree FMT recipients. Germfree recipients are the most suitable model to investigate single bacteria or to investigate the interaction between a few bacteria, since no other micro-organisms are present in the recipients. To understand the effects of a complex microbiota composition on a physiological process or disease cross-fostering or pretreatment of mice with broad-spectrum antibiotics is more suitable since these models are a better reflection of normal physiology.

Identification of bacteria causally involved in disease

Although involvement of the microbiota in disease development has been clearly proven and is widely accepted, identification and validation of specific bacterial strains that contribute to disease development is a major challenge in the microbiota field and a necessity to start targeting the gut microbiota as a therapeutic target. Surana et al recently published a novel method to help address this problem. Specific pathogen free (SPF) mice or gnotobiotic mice harboring either mouse microbiota (MMb) or human microbiota (HMb)(Chung et al., 2012) compositions showed

a different susceptibility to develop colitis following administration of dextran sodium sulfate (DSS)(Surana and Kasper, 2017). SPF mice and HMb mice were protected against development of DSS-induced colitis with respect to MMb mice. To identify which bacteria could contribute to the differences in DSS-induced colitis susceptibility of MMb and HMb mice, MMb and HMb mice were cohoused for 1 or 3 days to produce hybrid microbiota compositions. Cohousing was protective against development of DSS-induced colitis and increasing the length of cohousing lead to a larger survival following DSS-induced colitis. Making use of pairwise comparisons between MMb, HMb, SPF mice and mice with hybrid microbiota compositions led to the discovery that only the taxon Lachnospiraceae was significantly different between all pair-wise comparisons, indicating the relevance of the taxon Lachnospiraceae for development of colitis (Surana and Kasper, 2017). Thereby bacterial candidates with a protective effect were narrowed down to one bacterial family. Feces of the Lachnospiraceae rich HMb microbiota were consequently cultured on a semi-selective medium, to enrich for Lachnospiraceae. Combining 16SrDNA sequencing, MALDI-TOF analysis and biochemical tests identified the novel bacterial species Clostridium Immunis, from the family Lachnospiraceae as the bacterial strain responsible for the protective effect of the HMb microbiota against DSS-induced colitis. Thus, making use of a cohousing approach to generate hybrid microbiotas and applying multiple pair-wise comparisons of microbiota compositions combined with disease phenotype information is an interesting approach to narrow down the number of candidate bacteria. Combining culturing techniques with next-generation sequencing approaches, biochemical assays and MALDI-TOF analysis can consequently be used to identify the individual bacterial species within the candidate taxonomies. Although this is an interesting and promising approach to start understanding which bacteria are involved in disease development, this approach does rely on the ability to culture bacteria from gut microbiota. Until now difficulties to culture strictly anaerobic bacteria has hampered the ability to identify and causally relate bacterial strains to disease development. This limitation has been recognized by multiple groups in the field and efforts to increase the number of culturable bacteria from the gut microbiome are ongoing (Browne et al., 2016; Lau et al., 2016; Rajilić-Stojanović and de Vos, 2014). Comparing classical culturing

techniques with 16S rRNA sequencing of the gut microbiome estimated that 76% of the OTUs observed by culture-independent techniques can be cultured by combining 66 different culturing conditions(Lau et al., 2016). Although this is a promising result, the major limitation of this study and others is the usage of reference-based OTU-picking during 16S sequencing analysis. During reference-based OTU-picking, the OTUs that are not part of the selected reference database are discarded, leading to an underestimation of the bacteria present in the gut microbiome. Since this serves as the culture-independent reference for total bacteria present in the gut microbiome, this will lead to an overestimation of the proportion of bacteria that can be cultured ex vivo. In addition, the resolution of 16S rRNA sequencing is limited and taxonomies are hardly ever identified on the level of bacterial strains. Usage of metagenomics shotgun sequencing has greatly improved the resolution and is able to identify taxonomies from the gut microbiome onto the strain level. More importantly, metagenomics shotgun sequencing also gives insight into function of bacteria by mapping the different genes of all different taxonomies identified. This information can be used to explore which culturing conditions may be successful to capture the full complexity of the gut microbiome. Overall, efforts made until now will help to detect the bacteria that functionally attribute to or protect against disease, it is however necessary to further explore different combinations of culturing conditions. Metagenomic shotgun sequencing can support this process by implementing bacterial gene information to further optimize ex vivo culturing. Furthermore, more accurate estimates of percentages of bacteria from the human gut microbiome that can be cultured ex vivo can be retrieved by comparing the numbers of ex vivo-cultured bacteria to a reference dataset retrieved from the fecal samples of the same individuals by open-reference OTU-picking of metagenomics shotgun sequencing samples. These developments will be a necessary step to move from association to causality and to start understanding the complex mechanisms by which the microbiota interact with each other and their host and how these interactions contribute to physiology and disease development. Ultimately understanding the complex mechanisms in this ecosystem can lead to identification of beneficial or harmful bacteria in a specific microbiota composition for a specific disease, thereby facilitating the ability for development of microbiota targeted therapies in the future.

Concluding remarks

The field of microbiota research in the development of cardiovascular diseases is a fascinating research field with a lot of potency to discover novel mechanisms that are involved in the development of cardiovascular disease. Identifying bacteria or bacterial metabolites that individually or in congregation with other bacteria or metabolites influence the development of cardiovascular disease is however still a major challenge. Understanding the complex interactions between the many bacteria present in the microbiota and human physiology will be instrumental to eventually use the gut microbiota as a target for treatment of cardiovascular disease. Our studies describe a causal role of the gut microbiota in atherogenesis and highlight the importance of gut microbiota composition and localization as well as the intestinal immune barrier in atherogenesis. Furthermore, our studies suggest that the interaction between the intestinal immune barrier, gut microbiota composition and localization influence the development of cardiovascular disease. Our studies warrant follow-up research to validate and specify the suggested mechanisms, to identify which bacteria or bacterial metabolites are important in atherogenesis and to understand whether the role of specific bacteria or bacterial metabolites is dependent on the composition of the gut microbiota.

References

Aguilar, E.C., Leonel, A.J., Teixeira, L.G., Silva, A.R., Silva, J.F., Pelaez, J.M.N., Capettini, L.S.A., Lemos, V.S., Santos, R.A.S., and Alvarez-Leite, J.I. (2014). Butyrate impairs atherogenesis by reducing plaque inflammation and vulnerability and decreasing NFκB activation. Nutr. Metab. Cardiovasc. Dis. 24, 606–613.

Atarashi, K., Tanoue, T., Shima, T., Imaoka, A., Kuwahara, T., Momose, Y., Cheng, G., Yamasaki, S., Saito, T., Ohba, Y., et al. (2011). Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. Science (80-.). 331, 337–341.

Bäckhed, F., Ding, H., Wang, T., Hooper, L. V, Koh, G.Y., Nagy, A., Semenkovich, C.F., and Gordon, J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage. Proc. Natl. Acad. Sci. U. S. A. 101, 15718–15723.

Brandsma, E., Houben, T., Fu, J., Shiri-Sverdlov, R., and Hofker, M.H. (2015). The immunity-diet-microbiota axis in the development of metabolic syndrome. Curr. Opin. Lipidol. 26, 73–81.

Browne, H.P., Forster, S.C., Anonye, B.O., Kumar, N., Neville, B.A., Stares, M.D., Goulding, D., and Lawley, T.D. (2016). Culturing of "unculturable" human microbiota reveals novel taxa and extensive sporulation. Nature 533, 543–546.

Cani, P.D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A.M., Delzenne, N.M., and Burcelin, R. (2008). Changes in Gut Microbiota Control Metabolic Endotoxemia-Induced Inflammation in High-Fat Diet–Induced Obesity and Diabetes in Mice. Diabetes 57, 1470–1481.

Caricilli, A.M., Picardi, P.K., de Abreu, L.L., Ueno, M., Prada, P.O., Ropelle, E.R., Hirabara, S.M., Castoldi, Â., Vieira, P., Camara, N.O.S., et al. (2011). Gut Microbiota Is a Key Modulator of Insulin Resistance in TLR 2 Knockout Mice. PLoS Biol 9, e1001212.

Carter, D.B., Kennett, M.J., and Franklin, C.L. (2002). Use of perphenazine to control cannibalism in DBA/1 mice. Comp. Med. 52, 452–455.

Celaj, S., Gleeson, M.W., Deng, J., O'Toole, G.A., Hampton, T.H., Toft, M.F., Morrison, H.G., Sogin, M.L., Putra, J., Suriawinata, A.A., et al. (2014). The microbiota regulates susceptibility to Fas-mediated acute hepatic injury. Lab. Investig. 94, 938–949.

Chowdhury, S.R., King, D.E., Willing, B.P., Band, M.R., Beever, J.E., Lane, A.B., Loor, J.J., Marini, J.C., Rund, L.A., Schook, L.B., et al. (2007). Transcriptome profiling of the small intestinal epithelium in germfree versus conventional piglets. BMC Genomics 8, 215.

Chung, H., Pamp, S.J., Hill, J.A., Surana, N.K., Edelman, S.M., Troy, E.B., Reading, N.C., Villablanca, E.J., Wang, S., Mora, J.R., et al. (2012). Gut Immune Maturation Depends on Colonization with a Host-Specific Microbiota. Cell 149, 1578–1593.

Corrêa-Oliveira, R., Fachi, J.L., Vieira, A., Sato, F.T., and Vinolo, M.A.R. (2016). Regulation of immune cell function by short-chain fatty acids. Clin. Transl. Immunol. 5, e73.

Couturier-Maillard, A., Secher, T., Rehman, A., Normand, S., De Arcangelis, A., Haesler, R., Huot, L., Grandjean, T., Bressenot, A., Delanoye-Crespin, A., et al. (2013). NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. J. Clin. Invest. 123, 700–711.

Cox, M.A., Jackson, J., Stanton, M., Rojas-Triana, A., Bober, L., Laverty, M., Yang, X., Zhu, F., Liu, J., Wang, S., et al. (2009). Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E(2) and cytokines. World J. Gastroenterol. 15, 5549–5557.

Craciun, S., and Balskus, E.P. (2012). Microbial conversion of choline to trimethylamine requires a glycyl radical enzyme. Proc. Natl. Acad. Sci. U. S. A. 109, 21307–21312.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A. V., Devlin, A.S., Varma, Y., Fischbach, M.A., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature 505, 559–563.

Denning, T.L., Norris, B.A., Medina-Contreras, O., Manicassamy, S., Geem, D., Madan, R., Karp, C.L., and Pulendran, B. (2011). Functional Specializations of Intestinal Dendritic Cell and Macrophage Subsets That Control Th17 and Regulatory T Cell Responses Are Dependent on the T Cell/APC Ratio, Source of Mouse Strain, and Regional Localization. J. Immunol. 187, 733.

Devkota, S., Wang, Y., Musch, M.W., Leone, V., Fehlner-Peach, H., Nadimpalli, A., Antonopoulos, D.A., Jabri, B., and Chang, E.B. (2012). Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature 487, 104–108.

Ding, S., Chi, M.M., Scull, B.P., Rigby, R., Schwerbrock, N.M.J., Magness, S., Jobin, C., and Lund, P.K. (2010). High-fat diet: bacteria interactions promote intestinal inflammation which precedes and correlates with obesity and insulin resistance in mouse. PLoS One 5, e12191.

Elinav, E., Strowig, T., Kau, A.L., Henao-Mejia, J., Thaiss, C.A., Booth, C.J., Peaper, D.R., Bertin, J., Eisenbarth, S.C., Gordon, J.I., et al. (2011). NLRP6 Inflammasome Regulates Colonic Microbial Ecology and Risk for Colitis. Cell 145, 745–757.

Emoto, T., Yamashita, T., Sasaki, N., Hirota, Y., Hayashi, T., So, A., Kasahara, K., Yodoi, K., Matsumoto, T., Mizoguchi, T., et al. (2016). Analysis of Gut Microbiota in Coronary Artery Disease Patients: a Possible Link between Gut Microbiota and Coronary Artery Disease. J. Atheroscler. Thromb. 23, 908–921.

Ericsson, A.C., and Franklin, C.L. (2015). Manipulating the gut microbiota: Methods and challenges. ILAR J. 56, 205–217.

Fu, J., Bonder, M.J., Cenit, M.C., Tigchelaar, E.F., Maatman, A., Dekens, J.A.M., Brandsma, E., Marczynska, J., Imhann, F., Weersma, R.K., et al. (2015). The Gut Microbiome Contributes to a Substantial Proportion of the Variation in Blood Lipids. Circ. Res. 117, 817–824.

Fuhrer, A., Sprenger, N., Kurakevich, E., Borsig, L., Chassard, C., and Hennet, T. (2010). Milk sialyllactose influences colitis in mice through selective intestinal bacterial colonization. J. Exp. Med. 207, 2843–2854.

Gaboriau-Routhiau, V., Rakotobe, S., Lécuyer, E., Mulder, I., Lan, A., Bridonneau, C., Rochet, V., Pisi, A., De Paepe, M., Brandi, G., et al. (2009). The Key Role of Segmented Filamentous Bacteria in the Coordinated Maturation of Gut Helper T Cell Responses. Immunity 31, 677–689.

Garrett, W.S., Lord, G.M., Punit, S., Lugo-Villarino, G., Mazmanian, S.K., Ito, S., Glickman, J.N., and Glimcher, L.H. (2007). Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell 131, 33–45.

Gregory, J.C., Buffa, J.A., Org, E., Wang, Z., Levison, B.S., Zhu, W., Wagner, M.A., Bennett, B.J., Li, L., DiDonato, J.A., et al. (2015). Transmission of Atherosclerosis Susceptibility with Gut Microbial Transplantation. J. Biol. Chem. 290, 5647–5660.

Gurav, A., Sivaprakasam, S., Bhutia, Y.D., Boettger, T., Singh, N., and Ganapathy, V. (2015). Slc5a8, a Na^+-coupled high-affinity transporter for short-chain fatty acids, is a conditional tumour suppressor in colon that protects against colitis and colon cancer under low-fibre dietary conditions. Biochem. J. 469, 267–278.

Han, D., Walsh, M.C., Kim, K.S., Hong, S.-W., Lee, J., Yi, J., Rivas, G., Surh, C.D., and Choi, Y. (2015). Microbiota-Independent Ameliorative Effects of Antibiotics on Spontaneous Th2-Associated Pathology of the Small Intestine. PLoS One 10, e0118795.

Hansson, G.K. (2017). Inflammation and Atherosclerosis - The End of a Controversy. Circulation CIRCULATIONAHA.117.030484.

Helgeland, L., Vaage, J.T., Rolstad, B., Midtvedt, T., and Brandtzaeg, P. (1996). Microbial colonization influences composition and T-cell receptor V beta repertoire of intraepithelial lymphocytes in rat intestine. Immunology 89, 494–501.

Hemmi, H., and Akira, S. (2005). TLR signalling and the function of dendritic cells. Chem. Immunol. Allergy 86, 120–135.

Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W.Z., Strowig, T., Thaiss, C.A., Kau, A.L., Eisenbarth, S.C., Jurczak, M.J., et al. (2012). Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. Nature 482:179–185.

Hill, D.A., Hoffmann, C., Abt, M.C., Du, Y., Kobuley, D., Kirn, T.J., Bushman, F.D., and Artis, D. (2010). Metagenomic analyses reveal antibiotic-induced temporal and spatial changes in intestinal microbiota with associated alterations in immune cell homeostasis. Mucosal Immunol. 3, 148–158.

Hooper, L. V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I. (2001). Molecular Analysis of Commensal Host-Microbial Relationships in the Intestine. Science (80-.). 291, 881–884.

Hufeldt, M.R., Nielsen, D.S., Vogensen, F.K., Midtvedt, T., and Hansen, A.K. (2010). Variation in the gut microbiota of laboratory mice is related to both genetic and environmental factors. Comp. Med. 60, 336–347.

Ichinohe, T., Pang, I.K., Kumamoto, Y., Peaper, D.R., Ho, J.H., Murray, T.S., and Iwasaki, A. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc. Natl. Acad. Sci. 108, 5354–5359.

Ivanov, I.I., Frutos, R.D.L., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B., and Littman, D.R. (2008). Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe 4, 337–349.

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S. V., et al. (2009). Induction of Intestinal Th17 Cells by Segmented Filamentous Bacteria. Cell 139, 485–498.

Jie, Z., Xia, H., Zhong, S.-L., Feng, Q., Li, S., and Liang, S. (2017). The gut microbiome in atherosclerotic cardiovascular disease. Nat. Commun. 1–11.

Karlsson, F.H., Fåk, F., Nookaew, I., Tremaroli, V., Fagerberg, B., Petranovic, D., Bäckhed, F., and Nielsen, J. (2012). Symptomatic atherosclerosis is associated with an altered gut metagenome. Nat. Commun. 3, 1245.

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., and Bäckhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. Cell 165, 1332–1345.

Lau, J.T., Whelan, F.J., Herath, I., Lee, C.H., Collins, S.M., Bercik, P., and Surette, M.G. (2016). Capturing the diversity of the human gut microbiota through culture-

enriched molecular profiling. Genome Med. 8, 72.

Lewis, S.J., and Heaton, K.W. (1997). Increasing butyrate concentration in the distal colon by accelerating intestinal transit. Gut 41, 245–251.

Li, J., Lin, S., Vanhoutte, P.M., Woo, C.W., and Xu, A. (2016). Akkermansia muciniphila protects against atherosclerosis by preventing metabolic endotoxemia-induced inflammation in Apoe-/- Mice. Circulation 133, 2434–2446.

Libby, P., Ridker, P.M., and Hansson, G.K. (2011). Progress and challenges in translating the biology of atherosclerosis. Nature 473, 317–325.

Liu, L., Li, L., Min, J., Wang, J., Wu, H., Zeng, Y., Chen, S., and Chu, Z. (2012). Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells. Cell. Immunol. 277, 66–73.

Luck, H., Tsai, S., Chung, J., Clemente-Casares, X., Ghazarian, M., Revelo, X.S., Lei, H., Luk, C.T., Shi, S.Y., Surendra, A., et al. (2015). Regulation of Obesity-Related Insulin Resistance with Gut Anti-inflammatory Agents. Cell Metab. 21, 527–542.

Martinez-Medina, M., Denizot, J., Dreux, N., Robin, F., Billard, E., Bonnet, R., Darfeuille-Michaud, A., and Barnich, N. (2014). Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut 63, 116–124.

Millard, A.L., Mertes, P.M., Ittelet, D., Villard, F., Jeannesson, P., and Bernard, J. (2002). Butyrate affects differentiation, maturation and function of human monocyte-derived dendritic cells and macrophages. Clin. Exp. Immunol. 130, 245–255.

Muniz, L.R., Knosp, C., and Yeretssian, G. (2012). Intestinal antimicrobial peptides during homeostasis, infection, and disease. Front. Immunol. 3, 310.

Nemoto, H., Kataoka, K., Ishikawa, H., Ikata, K., Arimochi, H., Iwasaki, T., Ohnishi, Y., Kuwahara, T., and Yasutomo, K. (2012). Reduced diversity and imbalance of fecal microbiota in patients with ulcerative colitis. Dig. Dis. Sci. 57, 2955–2964.

Park, J., Kim, M., Kang, S.G., Jannasch, A.H., Cooper, B., Patterson, J., and Kim, C.H. (2015). Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. Mucosal Immunol. 8, 80–93.

Rajilić-Stojanović, M., and de Vos, W.M. (2014). The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol. Rev. 38, 996–1047.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R.

(2004). Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell 118, 229–241.

Ridaura, V.K., Faith, J.J., Rey, F.E., Cheng, J., Duncan, A.E., Kau, A.L., Griffin, N.W., Lombard, V., Henrissat, B., Bain, J.R., et al. (2013). Gut Microbiota from Twins Discordant for Obesity Modulate Metabolism in Mice. Science (80-.). 341, 1241214.

Ridker, P.M., Everett, B.M., Thuren, T., MacFadyen, J.G., Chang, W.H., Ballantyne, C., Fonseca, F., Nicolau, J., Koenig, W., Anker, S.D., et al. (2017). Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N. Engl. J. Med. 377:1119-1131.

Schirmer, M., Smeekens, S.P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E.A., Jansen, T., Jacobs, L., Bonder, M.J., Kurilshikov, A., et al. (2016). Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. Cell 167, 1125–1136.e8.

Senthong, V., Li, X.S., Hudec, T., Coughlin, J., Wu, Y., Levison, B., Wang, Z., Hazen, S.L., and Tang, W.H.W. (2016). Plasma Trimethylamine N-Oxide, a Gut Microbe–Generated Phosphatidylcholine Metabolite, Is Associated With Atherosclerotic Burden. J. Am. Coll. Cardiol. 67, 2620–2628.

Siegmund, B. (2010). Interleukin-18 in intestinal inflammation: friend and foe? Immunity 32, 300–302.

Singh, N., Gurav, A., Sivaprakasam, S., Brady, E., Padia, R., Shi, H., Thangaraju, M., Prasad, P.D., Manicassamy, S., Munn, D.H., et al. (2014). Activation of gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. Immunity 40, 128–139.

Sperandio, B., Fischer, N., and Sansonetti, P.J. (2015). Mucosal physical and chemical innate barriers: Lessons from microbial evasion strategies. Semin. Immunol. 27, 111–118.

Surana, N.K., and Kasper, D.L. (2017). Moving beyond microbiome-wide associations to causal microbe identification. Nature 552, 244–247.

Tang, W.H.W., Wang, Z., Levison, B.S., Koeth, R.A., Britt, E.B., Fu, X., Wu, Y., and Hazen, S.L. (2013). Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N. Engl. J. Med. 368, 1575–1584.

Taurog, J.D., Richardson, J.A., Croft, J.T., Simmons, W.A., Zhou, M., Fernández-Sueiro, J.L., Balish, E., and Hammer, R.E. (1994). The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. J. Exp. Med. 180, 2359–2364.

Tedelind, S., Westberg, F., Kjerrulf, M., and Vidal, A. (2007). Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: A study with relevance to inflammatory bowel disease. 13, 2826–2832.

Tiihonen, K., Ouwehand, A.C., and Rautonen, N. (2010). Effect of overweight on gastrointestinal microbiology and immunology: correlation with blood biomarkers. Br. J. Nutr. 103, 1070.

Tunaru, S., Kero, J., Schaub, A., Wufka, C., Blaukat, A., Pfeffer, K., and Offermanns, S. (2003). PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. Nat. Med. 9, 352–355.

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R., and Gordon, J.I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1131.

Vijay-Kumar, M., Aitken, J.D., Carvalho, F.A., Cullender, T.C., Mwangi, S., Srinivasan, S., Sitaraman, S. V., Knight, R., Ley, R.E., and Gewirtz, A.T. (2010). Metabolic Syndrome and Altered Gut Microbiota in Mice Lacking Toll-Like Receptor 5. Science (80-.). 328, 228–231.

Vinolo, M.A.R., Rodrigues, H.G., Nachbar, R.T., and Curi, R. (2011). Regulation of inflammation by short chain fatty acids. Nutrients 3, 858–876.

Wang, X., Ota, N., Manzanillo, P., Kates, L., Zavala-Solorio, J., Eidenschenk, C., Zhang, J., Lesch, J., Lee, W.P., Ross, J., et al. (2014). Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes. Nature 514, 237–241.

Wang, Z., Klipfell, E., Bennett, B.J., Koeth, R., Levison, B.S., DuGar, B., Feldstein, A.E., Britt, E.B., Fu, X., Chung, Y.-M., et al. (2011). Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472, 57–63.

Summary

Cardiovascular disease (CVD) causes 17.7 million deaths annually worldwide and is therefore a major health burden to our society. Over the last century, CVD treatment has greatly improved. The discovery of cholesterol lowering drugs such as statins and PCSK9 inhibitors has led to a 50% reduction in cardiovascular events, by reducing plasma cholesterol levels into normolipidemic ranges. However, atherosclerosis, the main underlying cause of CVD, is not solely a lipid-driven disease. Indeed, numerous studies, including the CANTOS trial, have demonstrated a critical role for inflammation in atherosclerosis and CVD risk. Understanding the factors that contribute to inflammation is therefore of great importance.

The gut microbiota is one such factor that has recently been linked to cardiovascular disease and inflammation. However, it is unknown whether alterations in microbiota composition can contribute to CVD development and if interactions between the gut immune barrier, diet and gut microbiota can affect systemic inflammation and atherogenesis. In this thesis, we aimed to understand how the interaction between the diet, gut microbiota and intestinal immune barrier contributes to systemic inflammation and atherosclerosis.

In **Chapter 2** we described how the gut microbiota is controlled by the mucosal immune system and how the development of non-alcoholic fatty liver disease (NAFLD), type 2 diabetes and CVD is related to aberrancies in gut microbiota control.

In **Chapter 3** we investigated the effect of a pro-inflammatory gut microbiota on the development of systemic inflammation and atherosclerosis. We used the gut microbiota of Caspase1 knockout (*Casp1*$^{-/-}$) mice, as previous studies have shown that alterations in their microbiota sensitize mice to the development of several inflammatory diseases, such as intestinal and hepatic inflammation. In addition, we used the hyperlipidemic low-density-lipoprotein receptor knockout (*Ldlr*$^{-/-}$) mice, a well-established model for atherosclerosis. We transplanted the gut microbiota of *Casp1*$^{-/-}$ mice into antibiotic treated *Ldlr*$^{-/-}$ mice and fed the mice a high-fat cholesterol (HFC) diet for 13 weeks. We found that *Casp1*$^{-/-}$ microbiota increased atherosclerosis in the aortic root of HFC-fed *Ldlr*$^{-/-}$ mice. Subsequently, we explored multiple mechanisms to understand how the pro-inflammatory microbiota of *Casp1*$^{-/-}$ mice did affect the process of atherogenesis. We observed that there was no

effect of the *Casp1*$^{-/-}$ microbiota on blood lipid levels. However, increased atherogenesis was accompanied by increased pro-inflammatory plasma cytokines, increased blood leukocyte numbers, particularly monocytes and neutrophils, neutrophil accumulation in atherosclerotic plaques and reduced levels of short-chain fatty acids (SCFAs) in the cecum. These results imply a causal relationship between microbiota composition, inflammation, and atherosclerosis and suggest that production of the anti-inflammatory SCFAs by the gut microbiota might be protective against atherosclerosis.

The production of mucus and excretion of antimicrobial peptides in the gut forms the first line of defense against infiltration of the gut microbiota into the intestinal epithelial barrier. Thus antimicrobial peptides such as REG3γ could play a protective role in atherogenesis by preventing the infiltration of the gut microbiota into the intestinal epithelial barrier. In **chapter 4** we investigated the contribution of the antimicrobial peptide REG3γ in the susceptibility to atherogenesis in *Reg3*γ knockout (*Reg3*γ$^{-/-}$) mice. To create a hyperlipidemic mouse model comparable to the atherosclerosis-prone *Ldlr*$^{-/-}$ mice, we overexpressed a PCSK9 gain-of-function mutant in the liver of female *Reg3*γ$^{-/-}$ and WT littermate mice via an AAV-delivery system and fed these mice a high-fat, high-cholesterol diet (HFC) for 11 weeks. Analysis of atherosclerotic lesion size in the aortic root demonstrated a 28% increase in lesion size in *Reg3*γ$^{-/-}$ (PCSK9) mice with respect to WT(PCSK9) littermates. We also observed that these effects were independent of alterations in blood lipid levels, intestinal permeability or microbiota composition. This shows that REG3γ plays a protective role against the development of atherosclerosis and indicates the importance of antimicrobial peptides produced in the gut in the protection against atherosclerosis development.

Intestinal inflammation leads to increased gut permeability thereby facilitating the transfer of bacteria from the lumen of the gut into the systemic circulation. This can consequently trigger low-grade systemic inflammation. 5-aminosalicylic acid (5-ASA) is widely used in the treatment of inflammatory bowel diseases and was recently shown to improve insulin resistance in diet-induced obese mice through its gut specific anti-inflammatory effect. In addition, cholesterol in western type diets has been suggested to be a driving factor for intestinal inflammation. Thus, in **Chapter 5** we investigated whether dietary cholesterol can

promote intestinal inflammation and accelerate the development of insulin resistance. For this, we fed wildtype mice a low-fat diet (LFD), high-fat diet (HFD) or a high-fat cholesterol diet (HFCD) supplemented with or without the anti-inflammatory agent 5-ASA. Although obesity and systemic glucose intolerance was induced by both HFD and HFCD feeding, intestinal barrier function was not compromised. Administration of the anti-inflammatory agent 5-ASA did not result in protection against obesity and systemic glucose intolerance. Thus, our study indicates that dietary fat or cholesterol does not directly affect intestinal barrier function in wildtype mice.

In conclusion, our studies describe a causal role of the gut microbiota in atherogenesis and highlight the importance of gut microbiota composition and localization as well as the intestinal immune barrier in atherogenesis. Furthermore, our studies suggest that the interaction between the intestinal immune barrier, gut microbiota composition and localization influence the development of CVD. Our studies warrant follow-up research to validate and specify the suggested mechanisms, to identify which bacteria or bacterial metabolites are important in atherogenesis and to understand whether the role of specific bacteria or bacterial metabolites is dependent on the presence or absence of other bacteria in the gut microbiota.

Samenvatting

Hart- en vaatziekten veroorzaken wereldwijd 17,7 miljoen doden per jaar en zijn daarmee een grote belasting voor onze gezondheidszorg. Gedurende de afgelopen eeuw is de behandeling van hart- en vaatziekten sterk verbeterd. De ontdekking van medicijnen die in staat zijn om de cholesterolwaarden in het bloed sterk te verlagen heeft geleid tot een 50% reductie in de incidentie van hart- en vaatziekten. Aderverkalking is de voornaamste onderliggende oorzaak van hart-en vaatziekten. Het is echter gebleken dat aderverkalking niet alleen gedreven wordt door verhoogde bloed cholesterolwaarden. Recentelijk heeft een grote humane studie aangetoond dat inflammatie ook een belangrijke bijdrage levert aan aderverkalking. Zodoende is het essentieel om beter te begrijpen welke factoren bijdragen aan inflammatie en aderverkalking. Eén van die factoren die hierbij mogelijk een rol zou kunnen spelen is de samenstelling van de verschillende bacteriën in de darm, ook wel microbiota genoemd. Het is echter onbekend of veranderingen in de microbiota inderdaad kunnen bijdragen aan aderverkalking en inflammatie en hoe interacties met het immuunsysteem en het dieet hieraan kunnen bijdragen. In dit proefschrift hebben we onderzocht hoe de interactie tussen dieet, microbiota en het immuunsysteem in de darm kunnen bijdragen aan inflammatie en aderverkalking.

In **hoofdstuk 2** beschrijven we de effecten van het darm immuunsysteem op de samenstelling van de microbiota in de darm en laten we zien hoe veranderingen in de samenstelling van de microbiota kunnen bijdragen aan type 2 diabetes, een vette lever en hart- en vaatziekten.

In **hoofdstuk 3** hebben we de effecten onderzocht van een pro-inflammatoire darm microbiota op de ontwikkeling van systemische inflammatie en aderverkalking. Om dit te onderzoeken hebben we allereerst gebruik gemaakt van een muismodel dat gevoelig is voor de ontwikkeling van aderverkalking. In deze muizen is het gen dat codeert voor de LDL-receptor uitgeschakeld (*Ldlr*$^{-/-}$ muizen) waardoor de bloedwaarde van het LDL-cholesterol in deze muizen verhoogd is en er aderverkalking optreedt. Daarnaast hebben we ook gebruik gemaakt van *Caspase1* knockout (*Casp1*$^{-/-}$) muizen. In deze muizen is het gen dat codeert voor caspase 1 uitgeschakeld. Het is bekend dat het uitschakelen van dit gen leidt tot een verandering in de samenstelling van de darm microbiota met ontstekingsreacties in de lever en de darmen tot gevolg.

Vervolgens hebben wij de darm microbiota van deze *Casp1*$^{-/-}$ muizen getransplanteerd naar *Ldlr*$^{-/-}$ muizen en hebben we deze muizen een hoog vet en cholesterol-rijk (HVC) dieet gegeven. Uit onze studie blijkt dat het introduceren van de pro-inflammatoire darm microbiota van *Casp1*$^{-/-}$ muizen in *Ldlr*$^{-/-}$ muizen tot een verergering in aderverkalking leidt. We hebben gezien dat veranderingen in aderverkalking niet afhankelijk zijn van veranderingen in de bloedwaarden van cholesterol en triglyceriden. De toename in aderverkalking ging echter wel gepaard met een verhoging in pro-inflammatoire signaalstoffen, ook wel cytokines genoemd. Verder vonden we ook een toename in het aantal witte bloedcellen in de circulatie, waarbij we specifiek een vermeerdering zagen in monocyten en neutrofielen. Meer neutrofielen werden ook gevonden in de atherosclerotische plaque (aderverkalking). Vervolgens hebben we onderzocht welke verandering in de samenstelling van de darm microbiota zou kunnen bijdragen aan de toename van systemische inflammatie en aderverkalking. We zagen een afname in het aantal bacteriën die korte keten vetzuren kunnen produceren, en dit ging gepaard met een afname van korte keten vetzuren in de darm. Van deze vetzuren is bekend dat ze inflammatie kunnen remmen. Onze resultaten impliceren een causaal verband tussen darm microbiota samenstelling, inflammatie en aderverkalking. Daarnaast suggereren onze data dat een grotere productie van de anti-inflammatoire korte keten vetzuren door de darm microbiota mogelijk een beschermende werking tegen aderverkalking heeft.

Het is bekend dat de productie van mucus (slijmlaag over de darm) en de productie van antimicrobiële eiwitten in de darm de eerste verdedigingslinie vormt tegen de infiltratie van microbiota in de darmwand. Echter, het is niet bekend of antimicrobiële eiwitten zoals REG3γ mogelijk een beschermende rol spelen bij het ontstaan van aderverkalking door te voorkomen dat microbiota uit de darm in de darmwand infiltreren. In **hoofdstuk 4** hebben we gebruik gemaakt van muizen waarin het gen dat codeert voor REG3γ is uitgeschakeld (*Reg3γ*$^{-/-}$ muizen) en vervolgens hebben we onderzocht wat de effecten hiervan zijn op de ontwikkeling van aderverkalking in deze muizen. Om *Reg3γ*$^{-/-}$ muizen gevoelig te maken voor aderverkalking hebben we deze muizen geïnjecteerd met een virus dat leidt tot een toename in de productie van het eiwit PCSK9 (*Reg3γ*$^{-/-}$ (PCKS9 muizen)). Hierdoor stijgen de LDL-cholesterol bloedwaarden en

neemt de gevoeligheid voor de ontwikkeling van aderverkalking in deze muizen aanzienlijk toe. Vervolgens hebben we de *Reg3γ*$^{-/-}$(PCSK9) muizen vergeleken met wildtype muizen (WT(PCSK9) muizen) die ook geïnjecteerd waren met hetzelfde PCSK9 virus en 11 weken lang hetzelfde HVC-dieet gegeten hadden. Na analyse van de atherosclerotische plaques zagen we dat de oppervlakte ervan 28% groter was in de *Reg3γ*$^{-/-}$(PCSK9) muizen ten opzichte van de controle muizen. Verder zagen we dat de toename in aderverkalking onafhankelijk was van de samenstelling van de darm microbiota, de cholesterolwaarden in het bloed en de doorlaatbaarheid van de darmwand. Dit laat zien dat het antimicrobiële eiwit REG3γ een beschermende rol speelt bij de ontwikkeling van aderverkalking en benadrukt het belang van de productie van antimicrobiële eiwitten in de darm bij de bescherming tegen aderverkalking.

Inflammatie in de darmen leidt tot een verhoogde doorlaatbaarheid van de darmen, waardoor bacteriën uit het lumen van de darm in het bloed terecht kunnen komen. Dit kan vervolgens bijdragen aan laaggradige systemische inflammatie. Het medicijn 5-aminosalicylic acid (5-ASA) wordt veelal gebruikt voor de behandeling van inflammatoire darmziekten. De consumptie van een vetrijk dieet (HV-dieet) kan bijdragen aan een verhoogde doorlaatbaarheid van de darmen. Recentelijk is ook met een muizen studie aangetoond dat 5-ASA een beschermend effect heeft tegen de ontwikkeling van type 2 diabetes. Dit beschermende effect werd veroorzaakt doordat toediening van 5-ASA voorkwam dat de doorlaatbaarheid van de darmen toenam na consumptie van een HV-dieet. Hierdoor werd voorkomen dat bacteriën of bacteriële fragmenten door de darmwand heen lekken en in het bloed terecht komen. Als gevolg hiervan kan er geen ontstekingsreactie ontstaan die op zijn beurt kan bijdragen aan een verergering van type 2 diabetes. Naast de hoeveelheid vet in een dieet wordt ook gedacht dat de aanwezigheid van cholesterol in het dieet eenzelfde effect kan hebben op de doorlaatbaarheid van de darm, inflammatie en type 2 diabetes. Zodoende hebben we in **hoofdstuk 5** de effecten van cholesterol in het dieet op de ontwikkeling van darminflammatie onderzocht en hebben we bekeken of dit kan leiden tot een versnelde ontwikkeling van type 2 diabetes. Om dit te onderzoeken hebben we WT muizen een laag vet (LV) dieet, HV-dieet of HVC-dieet gevoerd met of zonder toevoeging van het medicijn 5-ASA. Consumptie van zowel een HV- als HVC-dieet leidde tot een toename in systemische

glucose intolerantie, maar niet tot een toename in de doorlaatbaarheid van de darmen. Het anti-inflammatoire effect van het medicijn 5-ASA op de darmen had zodoende ook geen effect op de ontwikkeling van systemische glucose intolerantie. Onze studie impliceert dus dat vet en cholesterol in het dieet geen direct effect heeft op de doorlaatbaarheid van de darmen in wildtype muizen.

Alles samenvattend, tonen onze studies een causale rol aan van de darm microbiota bij de ontwikkeling van systemische inflammatie en aderverkalking. Verder benadrukken onze studies de beschermende werking van het immuunsysteem in de darmen en meer specifiek de productie van antimicrobiële eiwitten in de darm tegen hart- en vaatziekten. Verder roepen de resultaten van onze studies op tot nieuwe studies waarin meer inzicht verkregen moet worden in de specifieke bacteriën of metabolieten geproduceerd door bacteriën die bijdragen aan of beschermen tegen de ontwikkeling van hart- en vaatziekten.

Dankwoord

Eindelijk is het zover, het einde van mijn promotie-tijd komt nu echt in zicht. Met veel plezier heb ik de laatste jaren gewerkt aan mijn onderzoek om de rol van de bacteriën in de darm in de ontwikkeling van hart- en vaatziekten te ontrafelen. Ik heb de afgelopen jaren vele uren doorgebracht op het CDP en in het lab op de 4de verdieping van het ERIBA-instituut en ben in al die tijd geen enkele dag met tegenzin op mijn fiets/in de trein gestapt. Ik denk dat dit veel zegt over de fijne omgeving waarin ik mijn promotie-traject heb mogen doormaken. Het is natuurlijk niet altijd rozengeur en maneschijn geweest en ook ik ben tijdens mijn promotie regelmatig gefrustreerd geweest als dingen niet gingen zoals ik zelf graag wilde, maar de positieve sfeer en gezelligheid binnen de sectie moleculaire genetica hebben me altijd goed geholpen om me hier weer overheen te zetten en weer met frisse moed door te gaan.

Allereerst wil ik, hoewel het ergens een beetje gek voelt, heel graag Marten ontzettend bedanken. Beste Marten, vanaf dag 1 heb ik onze samenwerking als bijzonder prettig ervaren, jouw manier van begeleiden sloot erg goed aan op mijn stijl van werken. Je hebt me altijd veel ruimte gegeven om zelf dingen uit te zoeken en om mijn eigen ideeën te ontwikkelen. Deze ideeën bespraken we altijd tijdens ons gezamenlijke werkoverleg, dit waren altijd erg interessante gesprekken waarbij we allebei op een vrij directe manier aangaven wat we vonden van elkaars ideeën en standpunten. Voor een buitenstaander kan dit er best weleens uitgezien hebben als een verhitte discussie, persoonlijk heb ik dit echter altijd als heel leuk en leerzaam ervaren. Ondanks dat het er best stevig aan toe kon gaan waren de discussies altijd gericht op de inhoud en hadden we beide hetzelfde doel, namelijk het beter maken van de opzet van ons project of het aanscherpen van de onderzoeksvraag waar we ons op dat moment mee bezig hielden. Naast de inhoudelijke begeleiding van mijn projecten heb je je ook altijd erg bekommert om mijn persoonlijke ontwikkeling als wetenschapper. Met mijn achtergrond als biomedisch wetenschapper had ik weinig kaas gegeten van bioinformatica. Toen ik aangaf dat ik de microbiota analyses binnen mijn projecten niet wilde uitbesteden maar het belangrijk vond om dit zelf te kunnen kwam jij meteen met de suggestie om hier een geschikte cursus voor te zoeken. Het was natuurlijk veel efficiënter geweest om deze analyses uit te besteden, maar het heeft

mij als wetenschapper in het microbiota veld een stap verder gebracht dat ik datzelfde jaar de STAMPS-course in Woodshole heb gevolgd en daardoor in staat was om mijn eigen bioinformatica analyses uit te voeren. Dit is slechts 1 van de vele voorbeelden waaruit blijkt dat je niet alleen bezig was met de succesvolle uitvoering van mijn projecten, maar dat mijn ontwikkeling als wetenschapper minstens zo belangrijk was. In het begin van 2016 werd bij jou darmkanker geconstateerd waardoor we mijn promotietraject helaas niet samen af hebben kunnen maken. Ook tijdens je ziekbed ben je altijd betrokken gebleven bij jouw onderzoeksgroep en wilde je ook mij nog altijd graag van advies voorzien. Ik heb ontzettend veel bewondering voor je vermogen om anderen te blijven helpen toen je zelf al wist dat jij het niet zou gaan halen. Je bent dan ook een hele fijne promotor en vooral ook mentor geweest tijdens de eerste 2,5 jaar van mijn promotie, ontzettend bedankt!

Verder wil ik ook graag mijn copromotor Debby bedanken, gedurende mijn onderzoek onder de begeleiding van Marten raakte jij steeds meer geïnteresseerd in microbiota onderzoek en zodanig raakte ook jij betrokken bij mijn onderzoek. Toen Marten ziek werd en ik een nieuwe begeleider nodig had was het dan ook heel fijn dat jij de begeleiding kon overnemen om mijn promotie tot een goed einde te brengen. Je hebt me heel erg geholpen bij het structureren van mijn hoofdstukken door samen een goed plan te maken waar we gericht naar toe konden werken. Verder wil ik je ook graag bedanken voor alle hulp bij het schrijven van de hoofdstukken uit mijn proefschrift, je wist altijd feilloos aan te geven waar de verbeterpunten lagen en hoe deze aangepast konden worden.

Naast Debby wil ik ook graag Bart hartelijk bedanken, toen bleek dat Marten mij niet langer kon begeleiden stond ook jij meteen klaar om de begeleiding van mijn promotie over te nemen en heb jij de rol als promotor overgenomen. Je bent zelf natuurlijk gefascineerd door de werking van het COMMD-complex, maar was daarnaast ook zeer goed in staat om mij te helpen met mijn microbiota-onderzoek, jouw kritische blik, scherpe vragen en praktisch inhoudelijk kennis van de technieken in het lab hebben mij ontzettend geholpen tijdens mijn promotie en zijn van grote waarde geweest voor het ontstaan van dit proefschrift.

Daarnaast wil ik graag de leden van de leescommissie bedanken voor het lezen en beoordelen van mijn proefschrift. Prof. Dr. Bert Groen, Prof. Dr. Leo Joosten en Prof. Dr. Klaas-Nico Faber hartelijk dank voor

het goedkeuren van mijn proefschrift. Leo en Klaas-Nico ook ontzettend bedankt voor jullie betrokkenheid en nuttige bijdrages tijdens mijn promotie als leden van mijn PhD-commissie.
Beste Marit, Jing en Kuif, bedankt voor jullie kritische blik en scherpe vragen tijdens de besprekingen op maandag! Marit, heel erg bedankt voor je bijdrage middels je enorme kennis van het atherosclerose veld. Jing, heel erg bedankt voor je bijdrage op het gebied van microbiota analyses. Kuif, naast je enthousiasme voor onderzoek bleken we beide ook een passie te hebben voor fotografie, ook op dit gebied heb ik tijdens een ochtend in het mooie Mantingerbos in Drenthe nieuwe dingen van je geleerd, bedankt daarvoor.
Beste Paula en Marijke ook jullie wil ik graag bedanken, jullie kantoor was altijd een fijne plek om even te komen buurten (en om de snoeptrommel te plunderen natuurlijk). Om gewoon even gezellig een praatje te maken of om van jullie advies te krijgen over de keuzes die ik moest maken voor mijn toekomstige werk. Paula, jij hebt daarnaast ook ontzettend veel organisatorische taken op je genomen en me vaak uit de brand geholpen als er iets geregeld moest worden. Als ik jou vroeg of je me kon helpen was het nooit een probleem en vond jij altijd wel een oplossing om waar nodig door de bureaucratie heen te vechten om het voor elkaar te krijgen, bedankt daarvoor!
Beste Daphne, Henk, Marieke, Mirjam, Nicolette en Niels als groep van analisten hebben jullie ontzettend bijgedragen aan een fijne tijd tijdens mijn promotie. Jullie stonden als groep altijd klaar om te helpen, daardoor heb ik altijd het gevoel gehad dat ik in een team werkte en er nooit alleen voor stond. Als er technische of praktische problemen waren waar ik tegen aan liep dan kwam ik met jullie altijd wel tot een oplossing. Daarnaast namen jullie ook altijd de organisatie op je om iets leuks te organiseren voor mensen die afscheid namen of voor gezellige activiteiten zoals het jaarlijks terugkerende 'heel MolGen bakt'. Deze fijne sfeer heeft heel erg bijgedragen aan het plezier dat ik heb gehad tijdens mijn promotie. Daarnaast waren jullie ook altijd beschikbaar als het even niet ging zoals ik wou en ik gewoon even lekker wilde klagen. Mirjam, als vaste bewoner van het histolab met het bijbehorende foute uur (lees foute 24 uur) wil ik jou ook ontzettend bedanken voor al je hulp bij mijn atherosclerose experimenten. Je hebt mij met jouw handigheid op zowel het microtoom als de cryostaat ontzettend veel werk uit handen

genomen, bedankt daarvoor! Beste Nicolette, heel erg bedankt voor ondersteuning op het gebied van moleculaire technieken, wanneer ik advies nodig had over de productie van AAV of andere moleculaire technieken wist ik dat jij altijd klaar stond om te helpen. We hebben samen ook nog een paar mooie optredens gezien op Pinkpop waarbij het optreden van Bruno Mars met veel vuurwerkspektakel natuurlijk ons absolute hoogtepunt was. Beste Daphne, ook jij bent van grote waarde geweest voor mijn promotieonderzoek. Samen met jou en Niels heb ik vele uren doorgebracht in het CDP. Aan het begin moest je een beetje wennen aan mijn interesse in de darmen, met name het spoelen van de darmen in het CDP leidde (of misschien moet ik zeggen lijdde) bij jou in eerste instantie tot weinig enthousiasme. Die microbiota zoals dat zo hip heet vond jij maar een vieze bedoening. Desondanks was je altijd beschikbaar tijdens de opofferdagen en kan ik wel zeggen dat jij en Niels mijn dreamteam waren in het CDP. Je voelde het altijd perfect aan als Niels of ik even een handje extra nodig had en zorgde ervoor dat alles organisatorisch altijd strak geregeld was in het CDP en dat was geen overbodige luxe. Heel erg bedankt voor je bijdrage aan mijn projecten en vooral ook voor alle gezellige uurtjes op het CDP! Ik ben dan ook blij dat jij samen met Niels paranimf bent tijdens mijn promotie! Beste Niels, ik ben ook heel blij dat jij mij als paranimf helpt bij het voorbereiden van mijn promotie. Gedurende mijn promotie tijd hebben we heel veel tijd samen doorgebracht op het CDP. Ook jij hebt vanaf het begin altijd voor me klaargestaan. Dat begon al vroeg in mijn promotietraject, toen bleek dat we mijn muizen geen antibiotica konden geven via het drinkwater en ik daardoor moest besluiten om dit via 'gavage' toe te dienen. Er was alleen 1 probleem, ik had nog nooit een 'gavage' gedaan, gelukkig had jij veel ervaring bij het doen van proefdierexperimenten en heb je toen meteen tijd gemaakt om mij een spoedcursus 'orale gavage' te geven (helaas werden hier destijds nog geen diploma's voor uitgereikt). We zijn ook samen naar Amsterdam geweest om te leren aortaboogjes te isoleren, daar bleek meteen dat jij gouden handjes hebt. Terwijl ik me nog aan het oriënteren was op het opereren onder een microscoop had jij je aortaboogje al geïsoleerd. Jouw handigheid in proefdierwerk heeft je dan ook onmisbaar gemaakt voor mijn opofferdagen. Dit heeft er wel toe geleid dat de dagen dat je met mij het CDP in moest ergonomisch niet altijd even verantwoord waren, gelukkig was het echter wel altijd heel

gezellig! Tijdens deze dagen kon een lekker muziekje natuurlijk niet missen en we hadden dan ook graag een album van de Foo fighters, Nothing but Thieves of een andere rockband aan staan (en om Daphne ook tevreden te houden natuurlijk ook af en toe wat rustigere muziek). Verder heb ik natuurlijk ook mooie herinneringen aan de verscheidene toertochten die ik met jou en Bart op de racefiets heb gemaakt. Dit alles heeft heel erg bijgedragen aan mijn mooie tijd bij MolGen, bedankt daarvoor! Ook wil ik graag Niels Mulder bedanken, beste Niels heel erg bedankt voor je hulp bij het opzetten van de productie van mij AAV-experimenten! Verder wil ik graag mijn mede-promovendi bij MolGen bedanken, Ailine, Alina, Bibi, Fede, Melinde, Natalia, Liesbeth en Willem, bedankt voor de fijne tijd op E4. De sfeer onderling was altijd erg goed, we hebben met elkaar menig artikel ontleed in onze journal clubs. Daarnaast was iedereen altijd bereid om elkaar te helpen met experimenten als er een keer extra handjes nodig waren. Ik wist dat ik in geval van nood jullie altijd om hulp kon vragen in het lab, bedankt! Dear Ailine, as my co-microbiota researcher we shared a passion for the same scientific field, but we also had a lot of fun discussing the next rock festival that we were planning to visit. Dear Bibi, we started at approximately the same time at MolGen under the supervision of Marten, thank you for always being available to help out with R and for your positivity and kindness! Dear Fede, thank you for all the nice scientific discussions that we had, I truly admire your passion and unlimited enthusiasm for Science! Furthermore, I will never forget our moment of glory when we used your grandmother's recipe to steal the victory of the first edition of 'heel MolGen bakt' together with Antoine. Beste Melinde, ook jij stond altijd klaar om te helpen als het nodig was of om gewoon even gezellig te kletsen, met name op maandagochtend was de bespreking van onze wedstrijden van die week natuurlijk onmisbaar. Dear Natalia, thanks for making my time at E4 enjoyable, I will always remember you as the princess of Neuschwanstein. Dear Antoine, you joined the department as a postdoc in the last period of my PhD, I am happy to say that it was always a joy to have you as my neighbor, you were always in for a joke, social event or some 'pingpong'. Furthermore, you were very motivating with your enthusiasm for every new scientific discovery in our group and had a lot of trust in publishing these interesting stories, thank you!

Ik wil ook graag mijn collega's van de afdeling Genetica bedanken,

samen met jullie en de microbiota-onderzoekers van onze afdeling vormden wij de 'poepgroep', ontzettend bedank voor jullie input in mijn experimenten. In het speciaal wil ik graag Arnau en Marc-Jan bedanken, jullie waren altijd mijn bioinformatica-helpdesk, als ik even vastliep met de bioinformatica en hulp nodig had, losten jullie mijn problemen altijd binnen de kortste keren op, ontzettend bedankt daarvoor!
Verder wil ik graag mijn ouders en mijn broers en zus bedanken. Pap, Mam, Johan & Lidia, Hendrik, Iris & DJ ontzettend bedankt voor alle steun tijdens mijn promotietraject. Op de momenten dat het promoveren even tegenzat kon ik bij jullie altijd mijn verhaal kwijt en als ik ergens niet bij kon zijn door mijn onderzoek dan was hier altijd alle begrip voor, bedankt daarvoor! Ook wil ik graag mijn 'schoonfamilie' bedanken, Henk, Trudy, Monique & Niek, Mariël & Wout, Laureen & Sjoerd en Wendy, heel erg bedankt voor alle gezellige momenten en jullie interesse in en enthousiasme voor 'mijn' muizen. Ook wil ik graag mijn vrienden bedanken, beste Chris, Hans, Peter, Horst, Kelvin, Koen, Rik en Reinald, afgelopen jaren heb ik menig uurtje met jullie doorgebracht in de kroeg of tijdens ons jaarlijkse vriendenweekend in de jacuzzi, dit waren altijd mooie momenten van ontspanning na een drukke week op het lab en een goede manier om weer op te laden als dat nodig was, bedankt daarvoor!
Ten slotte wil ik graag Evelien bedanken. Lieve Evelien we hebben elkaar vlak voor het begin van mijn PhD-tijd leren kennen bij Veracles. Ik heb me kort daarna met veel enthousiasme op mijn onderzoek gestort. Dat enthousiasme leidde er wel eens toe dat ik wat later thuis kwam dan gepland, soms liep een experiment een 'kwartiertje' uit, als ik daarna 1,5 uur later thuiskwam vond jij dat nooit een probleem en was er daarna altijd tijd voor ontspanning en gezelligheid. We hebben samen ook vele mooie reizen gemaakt de afgelopen jaren, tijdens deze actieve vakanties met jou kon ik altijd even heerlijk mijn zinnen verzetten om me daarna weer met hernieuwde energie op mijn promotie te storten. Ook op de momenten dat mijn promotieonderzoek voor mij frustrerend was stond je altijd voor me klaar om de problemen te relativeren en me weer te motiveren om weer verder te gaan. Met name in de afrondende fase van mijn proefschrift ben ik niet altijd even gezellig geweest, maar bleef jij mij toch stimuleren en motiveren. Jouw nuchterheid, positieve houding en steun hebben me ontzettend geholpen in de afronding van mijn proefschrift, ontzettend bedankt hiervoor!

List of publications

A pro-inflammatory gut microbiome increases systemic inflammation and accelerates atherosclerosis
ET Brandsma, NJ Kloosterhuis, M Koster, DC Dekker, MJJ Gijbels, S van der Velden, M Ríos Morales, MJR van Faassen, MG Loreti, A de Bruin, J Fu, F Kuipers, BM Bakker, M Westerterp, MPJ de Winther, MH Hofker, AJA van de Sluis and DPY Koonen
Circulation Research 2019

Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity A Zhernakova, A Kurilshikov, MJ Bonder, EF Tigchelaar, M Schirmer, T Vatanen, Z Mujagic, A Vich, G Falony, S Vieira-Silva, J Wang, F Imhann, **ET Brandsma**, SA. Jankipersadsing, M Joossens, M Carmen Cenit, P Deelen, MA Swertz, RK Weersma, EJM Feskens, MG Netea, D Gevers, D Jonkers, L Franke, YS Aulchenko, C Huttenhower, J Raes, MH. Hofker, RJ Xavier, C Wijmenga, J Fu
Science (2016)

Antigen-independent effects of Cholera Toxin subunit B on pneumococcal colonization
K Kuipers, D Diavatopoulos, F van Opzeeland, E Simonetti, C van den Kieboom, M Kerstholt, M Borczyk, D van Ingen-Schenau, **ET Brandsma**, MG Netea, MI. de Jonge
J Infect Dis. (2016)

Sex impacts Th1 cells, Tregs, and DCs in both intestinal and systemic immunity in a mouse strain and location-dependent manner
M Elderman, A van Beek, ***ET Brandsma***, B de Haan, H Savelkoul, P de Vos, & M Faas,
Biology of sex differences (2016)

Oxidized LDL at the crossroads of immunity in non-alcoholic steatohepatitis)
T Houben, **ET Brandsma**, SM Walenbergh, MH Hofker, R Shiri-Sverdlov
Biochim Biophys Acta (2016)

The immunity-diet-microbiota axis in the development of metabolic syndrome
E.T Brandsma, T Houben, J Fu, R Shiri-Sverdlov, and MH Hofker
Current Opinion In Lipodology (2015)

The Gut microbiome contributes to a substantial proportion of the variation in blood lipids
J Fu, MJ Bonder, M Carmen Cenit, EF Tigchelaar, A Maatman, JAM Dekens, **ET Brandsma**, J Marczynska, F Imhann, RK Weersma, L Franke, TW Poon, RJ Xavier, D Gevers, MH. Hofker, C Wijmenga and A Zhernakova
Circulation (2015)

Lipid Sorting by Ceramide Structure from Plasma Membrane to ER for the Cholera Toxin Receptor Ganglioside GM1
JF Chinnapen, WT Hsieh, YM Welscher te, DE Saslowsky, L Kaoutzani, **ET Brandsma**, L D'auria, H Park, JS Wagner, KR Drake, MC Kang, T Benjamin, MD Ullman, CE Costello, AK Kenworthy, T Baumgart, RH Massol, WI Lencer.
Developmental Cell (2012)